ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

World Journal of *Gastroenterology*

World J Gastroenterol 2022 January 21; 28(3): 275-401

Published by Baishideng Publishing Group Inc

World Journal of Gastroenterology

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Weekly Volume 28 Number 3 January 21, 2022

World Journal of Gastroenterology

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ABOUT COVER

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AIMS AND SCOPE

The primary aim of *World Journal of Gastroenterology* (*WJG*, *World J Gastroenterol*) is to provide scholars and readers from various fields of gastroenterology and hepatology with a platform to publish high-quality basic and clinical research articles and communicate their research findings online. *WJG* mainly publishes articles reporting research results and findings obtained in the field of gastroenterology and hepatology and covering a wide range of topics including gastroenterology, hepatology, gastrointestinal endoscopy, gastrointestinal surgery, gastrointestinal oncology, and pediatric gastroenterology.

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The *WJG* is now indexed in Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, and Scopus. The 2021 edition of Journal Citation Report® cites the 2020 impact factor (IF) for *WJG* as 5.742; Journal Citation Indicator: 0.79; IF without journal self cites: 5.590; 5-year IF: 5.044; Ranking: 28 among 92 journals in gastroenterology and hepatology; and Quartile category: Q2. The *WJG*'s CiteScore for 2020 is 6.9 and Scopus CiteScore rank 2020: Gastroenterology is 19/136.

RESPONSIBLE EDITORS FOR THIS ISSUE

NAME OF JOURNAL
World Journal of Gastroenterology

ISSN
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

LAUNCH DATE
October 1, 1995

FREQUENCY
Weekly

EDITORS-IN-CHIEF
Andrzej S Tarnawski

EDITORIAL BOARD MEMBERS
http://www.wjgnet.com/1007-9327/editorialboard.htm

PUBLICATION DATE
January 21, 2022

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World J Gastroenterol 2022 January 21; 28(3): 275-289

DOI: 10.3748/wjg.v28.i3.275

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

FRONTIER

Endothelial cells and blood vessels are major targets for COVID-19-induced tissue injury and spreading to various organs

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Author contributions: Ahluwalia A and Tarnawski AS designed the overall concept, outline of the manuscript, and contributed to the writing and editing of the manuscript, illustrations, and review of literature, and approved the final version of manuscript. Ahluwalia A (amrita.ahluwalia@va.gov) and Tarnawski AS (atarnawski@yahoo.com) are co-corresponding authors.

Conflict-of-interest statement: No conflict of interest.

Country/Territory of origin: United States

Specialty type: Gastroenterology and hepatology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

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Abstract

The coronavirus disease 2019 (COVID-19) infected so far over 250 million people and caused the death of over 5 million worldwide. Aging, diabetes, and cardiovascular diseases, conditions with preexisting impaired endothelial functions predispose to COVID-19. While respiratory epithelium is the main route of virus entry, the endothelial cells (ECs) lining pulmonary blood vessels are also an integral part of lung injury in COVID-19 patients. COVID-19 not only affects the lungs and respiratory system but also gastrointestinal (GI) tract, liver, pancreas, kidneys, heart, brain, and skin. Blood vessels are likely conduits for the virus dissemination to these distant organs. Importantly, ECs are also critical for vascular regeneration during injury/lesions healing and restoration of vascular network. The *World Journal of Gastroenterology* has published in last two years over 67 outstanding papers on COVID-19 infection with a focus on the GI tract, liver, pancreas, *etc.*, however, the role of the endothelial and vascular components as major targets for COVID-19-induced tissue injury, spreading to various organs, and injury healing have not been sufficiently emphasized. In the present article, we focus on these subjects and on current treatments including the most recent oral drugs molnupiravir and paxlovid that show a dramatic, significant efficacy in controlling severe COVID-19 infection.

Key Words: Endothelial cells; Impaired endothelial function; Blood vessels; SARS-CoV-2; COVID-19; Cytokine storm

Received: November 15, 2021
Peer-review started: November 15, 2021
First decision: November 22, 2021
Revised: December 2, 2021
Accepted: January 11, 2022
Article in press: January 11, 2022
Published online: January 21, 2022

P-Reviewer: Watanabe A
S-Editor: Wu YXJ
L-Editor: A
P-Editor: Wu YXJ

Core Tip: The coronavirus disease 2019 (COVID-19) pandemic has enormous health care and economic impact on the entire world - infecting more than 250 million people in 213 countries and territories, causing death of more than 5 million (as of November 1, 2021). We comment here on some outstanding papers on COVID-19 published in *World Journal of Gastroenterology* and reviewed the important role of endothelium and blood vessels in COVID-19 infection. Endothelial cells and blood vessels are both the targets and a conduit for the spread of severe acute respiratory syndrome coronavirus 2 and play a critical role in COVID-19-induced tissue injury and dissemination to various organs. Pre-existing endothelial impaired function could make endothelial cells more sensitive to COVID-19 or at least COVID-19-induced impairment might be synergistic with pre-existing impairment. That could be one contributing factor explaining why older or diabetic patients have more severe responses to infection, since these conditions are already impacted impaired endothelial function.

Citation: Tarnawski AS, Ahluwalia A. Endothelial cells and blood vessels are major targets for COVID-19-induced tissue injury and spreading to various organs. *World J Gastroenterol* 2022; 28(3): 275-289
URL: https://www.wjgnet.com/1007-9327/full/v28/i3/275.htm
DOI: https://dx.doi.org/10.3748/wjg.v28.i3.275

BIOGRAPHY

Andrzej S Tarnawski, MD, PhD, DSc (Med), AGAF, FACG: Received MD degree, PhD (pathology) and DSc (gastroenterology) from the University Medical School, Krakow, Poland, and became Associate Professor & V-Chair, Dept of Gastroenterology at that University. After completing GI fellowship at the University of Missouri, Columbia, MO, United States he joined the University of California, Irvine, USA as Associate Professor (1982-1986) and full Professor (1986-present). He served as: V-chair and Associate Chair, American Gastroenterological Association (AGA)/EGD 1997-1999 and 2008-2010; Scientific Director, Shimoda Symposia on Mucosal Defense in Japan (8 times); Chair, Research Fora DDW/AGA annual meetings (1996-2011); Chair, Pasteur Institute Euroconference and Chair/Co-chair of 68 other International Symposia. Publications, presentations & grants: 373 full, peer reviewed publications (*Lancet*, *Nature Med*, *JCI*, *Gastroenterology*, *Hepatology*, *Gut*, *PNAS*, *FASEB J*, *Am J Pathol*, *Cellular Mol Gastro Hepatol*, *Am J Physiol*, *Am J Gastroenterol*, *Endoscopy*, *Cell Signal*, *Cells*, and others); 20 book chapters; 533 presentations at international & U.S. meetings; 20 peer reviewed funded grants (NIH, VA Merit Review 1984-present), 4 US patents. Clinical and Research interest: Injury and protection of GI mucosa; cellular and molecular mechanisms of gastric, duodenal and esophageal healing-role of growth factors, signaling pathways, angiogenesis, NSAIDs, prostaglandins and *Helicobacter pylori* toxins; aging gastric mucosa; confocal endomicroscopy and molecular imaging; gene therapy. Awarded prestigious academic honors (*e.g.*, Glaxo Intl. Res. Award, Athalie-Clarke Award, Merentibus Medal Award, Peregrinator of Science Awards, Andre Robert's Distinguished Award, Notable Biomedical Research Investigator Award). Memberships: AGA (Fellow), Am. College of Gastroenterology (Fellow), Brit. Soc. of Gastroenterology, Japanese Soc. of Gastroenterology (Honorary), Hungarian Soc. of Gastroenterology (Honorary), Am. Soc. for Investigative Pathology, Association of Am. Physicians (by election) and others. Editorial Boards - 6 scientific journals. Sixteen of his former trainees hold academic positions in US Medical Schools (4 being Chairs of Departments). Twenty of his former international trainees and/or associates hold academic positions abroad (France, Germany, Hungary, Japan, Poland, Sweden, Switzerland) (Figure 1A).

Amrita Ahluwalia, PhD: Research Scientist and coronavirus disease 2019 (COVID-19) Investigator, Veterans Affairs Long Beach Healthcare System, Long Beach, United States. Awarded PhD (Physiology) by the Medical Sciences Program, Indiana University Bloomington, IN, United States. Publications/presentations/grants: 60 peer-reviewed research publications (*PNAS*, *Am J Physiol* , *Gene Therapy*, *Endocrinology*, *Molecular Endocrinology etc.*); 95 presentations at International and US research

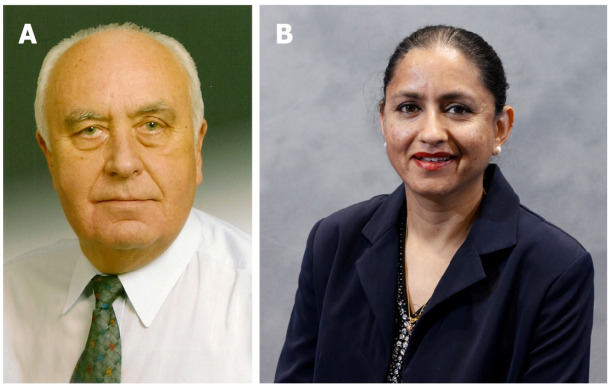

Figure 1 Authors. A: Andrzej S Tarnawski, Gastroenterology Research Department, University of California Irvine and the Veterans Administration Long Beach Healthcare System, Long Beach, CA 90822, United States; B: Amrita Ahluwalia, Research Service, Veterans Administration Long Beach Healthcare System, Long Beach, CA 90822, United States.

meetings; 6 peer-reviewed funded grants. Research Interests: Endothelial dysfunction, wound and tissue injury healing; gastroprotection; role of growth factors, angiogenesis, molecular imaging & gene therapy. Awards: Quest Diagnostics Young Investigator Award, AACR-AstraZeneca Scholar in Training Award, Robert W. Bullard Award - Outstanding Medical Science Student Award, Indiana University. Memberships: AGA, Am. Assoc. Physiology, Am. Heart Assoc.; Editorial Boards – 2 peer-reviewed scientific journals (Figure 1B).

INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic has had enormous health care and economic impact on the entire world - infecting more than 250 million people in 213 countries and territories, causing more than 5 million deaths (as of November 1, 2021). Its enormous magnitude is also reflected by an unprecedented number of publications related to COVID-19 so far approximate 210294 recorded in PubMed; 254358 recorded on PMC, and 3215 clinical trials just in 24 mo. These are staggering numbers compared to 47305 publications recorded on PubMed on *Helicobacter pylori (H. pylori)*– the world's most prevalent GI infection - published in about last 40 years.

COVID-19 is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and is highly infectious and transmitted by aerosol droplets. Therefore, it is not surprising that the respiratory tract including the lungs is the main affected organ by COVID-19 infection that leads to respiratory failure, hypoxia, multiorgan system failure and death. Numerous studies showed that COVID-19 not only affects the lungs and respiratory system but also the gastrointestinal tract (GI), liver, pancreas, kidneys, heart, brain, and skin[1-5]. SARS-CoV-2 RNA was detected in stool or rectal swabs in 34%-59% of infected patients[6]. The viral loads from stool samples peaked 2-3 wk after symptom onset and in some patients were detectable even after viral loads in the respiratory and/or sputum samples were not detectable[6]. The presence and persistence of viral RNA in the stool suggest the potential for enteric infection of SARS-CoV-2. This contention is supported by a study demonstrating that the GI tract is an alternative route for COVID-19 infection in the rhesus monkey model[7]. In that study, the authors showed that intranasal or gastric inoculation with SARS-CoV-2 induced infections and pathologic changes not only in respiratory tissues but also in digestive tissues[7]. In a recent letter to the *World Journal of Gastroenterology* (*WJG*) editor[8], Sica *et al*[8] contended that GI and hepatic involvement are the most common presenting symptoms of COVID-19 and multisystem inflammatory syndrome recently described in children and adolescents. This syndrome can lead to shock and multiple organ failure requiring intensive care[9].

Risk factors for COVID-19 severity include aging and comorbidities such as coronary artery disease, chronic kidney disease, hypertension, obesity, and diabetes [10-12], all of which exhibit preexisting endothelial dysfunction. However, the potential role of endothelial/vascular components as critical target sites for COVID-19-induced tissue injury and spreading to various organs, and the role of preexisting endothelial function impairment, *e.g.*, in aging or diabetes – conditions that facilitate

COVID-19 infection have not been sufficiently elaborated on. In the present article, we focus on these topics anticipating that providing a detailed information on endothelial cells (ECs) and vasculature in COVID-19 as critical targets may afford a better insight into the pathomechanism of this disease and add additional new therapies.

The SARS-CoV-2 virus spreads from its primary infection site (respiratory tract) to more distant organs indicating the involvement of ECs and blood vessels for disseminating infection. This contention is supported by some studies demonstrating the presence of SARS-CoV-2-like particles in ECs in several tissues *e.g.*, lung, kidneys, brain, and skin and observation that the clinical course of COVID-19 may include vascular complications such as thrombosis of blood vessels and thromboembolism[3,5,13-16].

The *WJG* has published in the last two years over 67 outstanding papers related to COVID-19 infection with a focus on GI tract and liver. These papers - original papers, retrospective studies and review articles on the pathophysiology, mechanisms, and clinical aspects and manifestations of COVID-19 related diseases of the digestive system including GI tubular system, liver, pancreas provided important information for the gastroenterologists, hepatologists, surgeons, researchers, pharmacologists, and clinicians. These papers provide information on the mechanisms of COVID-19 related tissue damage; the effects of immunosuppression in patients with inflammatory bowel disease and chronic liver disease; and the impact of COVID-19 on GI emergencies, endoscopy, diagnosis and treatments. These *WJG* articles were frequently viewed on the *WJG* website and cited in high-impact journals. We wish to point out one important paper by P. Samantha and AR Ghosh: "Environmental perspectives of COVID-19 outbreaks: A review" published in *World J Gastroenterol*. 2021 Sep 21;27(35):5822-585"[17]. In this paper the authors provided extensive information from an environmental perspective on the origin and current status of COVID-19[17] and summarized the geographical distribution of COVID-19 around the world including specific countries. They also elaborated on the details of coronavirus genus, species and receptors, virus susceptibility and incubation period, and summarized SARS-CoV-2 pathogenesis, the role of angiotensin-converting enzyme 2 (ACE2), the longevity of SARS-CoV-2 virus in the environment, meteorological influences, air quality and social impact. They emphasized that aging, cardiovascular diseases and diabetes predispose to COVID-19. The authors stressed that while drugs such as remdesivir, tocilizumab, lopinavir-ritonavir, azithromycin, *etc.*, are used in COVID-19 patients these drugs do not induce full recovery. The statement that there is no truly effective drug aimed at the causative agent, SARS-CoV-2 is no longer valid. On November 4 and 5, 2021 the released results of most recent clinical trials for COVID-19 treatments demonstrated that oral drugs inhibiting viral replication – Molnupiravir (Merck), and Paxlovid (Pfizer) showed very impressive efficacy in controlling severe COVID-19 infection. The interim analysis of the latter drug showed a dramatic approximate 90% reduction in risk of - hospitalization or death from COVID-19 compared to placebo in patients treated within three - five days of symptom onset. Most likely the vascular component of the disease was important part of this dramatic reduction.

Regarding COVID-19 pathomechanism, the potential role of endothelial and vascular components as critical target sites for COVID-19-induced tissue injury and spreading to various organs and the role of preexisting endothelial function impairment, *e.g.*, aging gastropathy has not been sufficiently emphasized. In this editorial article, we focus on the role vascular endothelium and blood vessels in COVID-19 infection (Table 1).

Increasing evidence suggests the essential role of endothelium and vasculature, in addition to the epithelial cells, in COVID-19 infection as a critical targets for SARS-CoV-2 and the resulting cytokine storm, and as the main effector for the pro-inflammatory and pro-coagulant state in COVID-19 patients[18,27-30]. Focus on ECs and vasculature in COVID-19 may also add additional insight into COVID-19 injury, its healing and tissue regeneration, and new therapies that impact endothelium and the blood vessels.

Although SARS-CoV-2 primarily targets the respiratory and alveolar epithelium, the high incidence of vascular complications in COVID-19 patients suggests that impaired function of ECs, which line the blood vessels and microvessels, may be critical factor in COVID-19 progression. SARS-CoV-2 causes endothelial dysfunction and thrombosis by two potential mechanisms: by directly infecting the endothelium, and disrupting its anti-thrombogenic and barrier properties, or indirectly by unleashing a local cytokine storm and systemic inflammatory response that results in endothelial injury (Table 2). Most likely, both these scenarios are in play in COVID-19.

Table 1 COVID-19 and endothelium/blood vessels

COVID-19 and endothelium/blood vessels
Endothelium and blood vessels are integral parts of COVID-19-induced tissue injury. Their injury is likely due to either direct viral infection and/or cytokine storm triggered by infection of adjacent epithelial cells and inflammatory response[18].
Blood vessels are critical for virus dissemination to distant organs.
Preexisting-impaired endothelial function, *e.g.*, in aging or diabetes are likely predisposing factors COVID-19. Our studies demonstrated that aging gastric mucosa has increased susceptibility to injury and prominent EC abnormalities (decreased VEGF, NGF and impaired mitochondrial function)[19-21].
ECs are critical for vascular regeneration (through angiogenesis and vasculogenesis) during injury/lesions healing and therefore are essential for the delivery of oxygen and nutrients to the healing site[22,23].
Several growth factors *e.g.*, NGF, IGF-1, HGF and BMD-stem cells may facilitate tissue regeneration in the healing phase[20,24,25].
Long-term effects of COVID-19, its vaccines and treatment on endothelium and vasculature remain to be determined.
Recently, new oral drugs inhibiting viral replication–Molnupiravir (Merck) and Paxlovid (Pfizer) showed significant efficacy in controlling severe COVID-19 infection by inhibiting viral replication. The interim analysis of the latter drug showed an 89% reduction in risk of COVID-19-related hospitalization or death from any cause compared to placebo in patients treated within three-five days of symptom onset[26].

COVID-19: Coronavirus disease 2019; EC: Endothelial cell; VEGF: Vascular endothelial growth factor; NGF: Nerve growth factor.

Table 2 Scenarios by which SARS-CoV-2 elicits endothelial damage

Scenario A: SARS-CoV-2 infection	Scenario B: Cytokine storm
SARS-CoV-2 infects and replicates within vascular ECs and new virus particles are released into the blood vessel. These virions can infect neighboring cells or are carried to distant organs *via* circulation	↑ IL-6, IL-1β, and TNFα release (cytokine storm) → endothelial damage ↑ vascular permeability → plasma extravasation ↑ vWF & FVIII (promote clot formation) and ↑ PAI-1 (inhibits clots lysis) → hypercoagulation

SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; IL: Interleukin; TNF: Tumor necrosis factor; vWF: von Willebrand factor.

Endothelium in normal and pathological conditions. Role in homeostasis, tissue repair and healing

The endothelium is a key player in vascular homeostasis[29,31-33]. ECs are critical for supplying oxygen and other nutrients to all cells and tissues, and are involved in coagulation and the generation of vasoactive substances, prostanoids, hormones and growth factors[33-38]. The unstimulated vascular endothelium is normally impermeable and acts as a selective barrier regulating exchange of fluids, nutrient delivery and waste removal while preventing entry of pathogens and harmful substances into the tissues. Microvessels consist of a single layer of thin (approximate 0.5-1 μm) ECs and occasional adherent cells such as pericytes[34-38]. The endothelial "barrier between neighboring ECs formed by prominent tight junctions prevents diffusion between cells. ECs act as a barrier between blood and the interstitial tissue, and regulate various physiological processes such as angiogenesis, inflammation, and immune response[31,35,36]. The endothelium contains special vesicles - Weibel-Palade bodies, which store various factors that regulate blood coagulation and leukocyte recruitment and extravasation such as von Willebrand factor (vWF), P-selectin, chemokines, interleukin-8, and eotaxin-3; endothelin-1, angiopoietin-2 and osteoprotegerin[39-42].

In response to local stimuli, ECs secrete endothelin and leukotriene C4 (potent vasoconstrictors), nitric oxide (NO) and prostacyclin (PGI2) (vasodilators) and empty the contents of the Weibel-Palade vesicles that affect the tone of vascular smooth muscle and result in neutrophil adhesion and/or other autocrine and/or paracrine actions. NO, prostacyclin, prostaglandin E2 (PGE2), carbon monoxide (CO), tissue plasminogen activator, vascular endothelial growth factor (VEGF) and bFGF are endothelial mediators that reduce platelet and leukocyte activation, prevent thrombi formation, promote thrombolysis, maintain tissue perfusion, and protect the microvascular wall against acute damage[33,36-38,43-46]. For example, our previous study demonstrated that 16,16 dimethyl PGE2 protects human gastric mucosa against injury by 40% ethanol by protecting and preserving integrity of endothelial cells of

gastric microvessels[47]. In response to wounding, infections or injurious stimuli, attachment between ECs is lost, resulting in increased endothelial permeability and edema[48].

The endothelium and blood vessels are integral parts of any tissue injury including COVID-19. Our previous studies demonstrated that ECs are critical targets of gastric mucosal injury by NSAIDs and ethanol, they initiate angiogenesis, and that age-related endothelial dysfunction of human and rat gastric endothelial cells results in impaired angiogenesis and delayed healing[19,20,24]. Our studies on aging gastropathy showed aging-related defects in ECs functions - angiogenesis, cell migration, proliferation, and healing of injury[19-21,49]. In a recent study, we also showed the critical role of mitochondria in aging gastric ECs; aging ECs have fewer mitochondria, and reduced mitochondrial membrane potential[50] that result in reduced ATP generation (Figure 2). We also demonstrated that treatment with VEGF and nerve growth factor (NGF) restores angiogenesis in cultured aging gastric ECs [20], accelerates healing of gastric ulcers and improves the quality of mucosal regeneration *in vivo* in aging rats[20,24].

Endothelial cells and COVID-19

SARS-CoV-2 is a single, positive-stranded RNA virus that uses a spike-protein (S-protein) expressed on its envelope to bind to the host cell's human protein receptor ACE2[51-53]. The human ACE2 protein was initially identified as ACE-related carboxypeptidase membrane-associated and secreted enzyme expressed predominantly on the endothelium of the human heart, kidney, and testis [54]. However, it is widely expressed in various cells and tissues[55]. SARS-CoV-2 employs the ACE2 receptor, transmembrane serine protease 2 (TMPRSS-2), and cathepsin B and L (CTSB, and CTSL) for infection[51-53,56,57]. SARS-CoV-2 was detected in the respiratory tract, kidneys, liver, heart, and brain (all of which are highly vascularized tissues) of infected individuals[55]. ECs, which line the blood vessels of all organs and maintain microvascular integrity, express the ACE2 receptor and the cellular proteases TMPRSS-2, CTSB, and CTSL[57]. ECs are, therefore, a target for SARS-CoV-2 and blood vessels likely route of this virus dissemination to various organs. Electron microscopy (EM) and histologic studies detected SARS-CoV-2 virus-like particles and proteins in ECs of the kidney, small bowel, lung, myocardium, skin, and brain[3,5,13-16]. Ackerman *et al*[15] showed abnormalities within the pulmonary microvasculature with congestion and micro-thrombi in lungs of COVID-19 patients, and visualized endothelial injury and lumen filled with cell fragments and degenerated organelles by electron microscopy. That study also showed increased ACE2-positive ECs and significant changes in endothelial morphology in lung autopsies of COVID-19 patients [15]. Varga *et al*[5] using EM evaluation reported evidence of viral particles in renal ECs of COVID-19 patients presenting with endotheliitis, which is an immune and inflammatory response within the endothelium of blood vessels.

Other studies visualized SARS-CoV-2 proteins in dermal and renal endothelium[13,58]. While some studies were not able to corroborate presence of SARS-CoV-2 in ECs of some tissues, there is strong evidence to support that SARS-CoV-2 infects ECs. Monteil *et al*[59] demonstrated that SARS-CoV-2 infects blood vessel organoids. SARS-CoV-2 virus particles range from approximate 70 to 120 nm[60-63]; therefore, in the absence of preexisting tissue injury, the virus would need to pass through the ECs to infect other tissues.

Endothelial dysfunction

The term endothelial dysfunction was originally used to identify the shift from a normal quiescent endothelium to an impaired endothelium with the inability to generate nitric oxide and other vasodilators. In a broader definition, endothelial dysfunction includes impairment of endothelial function (that we used for aging endothelium in our previous papers) - reduced angiogenesis, pro-inflammatory, pro-vasoconstriction, proliferative, and pro-coagulant phenotype[18,64-66]. In certain pathological conditions characterized by preexisting endothelial dysfunction, the ACE/Ang II axis is upregulated resulting in vasoconstriction, thrombosis, fibrosis, coagulopathy, and thrombophilia.

Endothelial dysfunction and endotheliitis in COVID-19

Emerging evidence indicates that preexisting endothelial dysfunction predisposes to COVID-19 infection and that COVID-19 induced endotheliitis further impairs endothelial integrity and function[27-30,32,34,67-76]. This is evidenced by the critical role of vascular endothelium in inflammation that results in dysregulation of cytokines

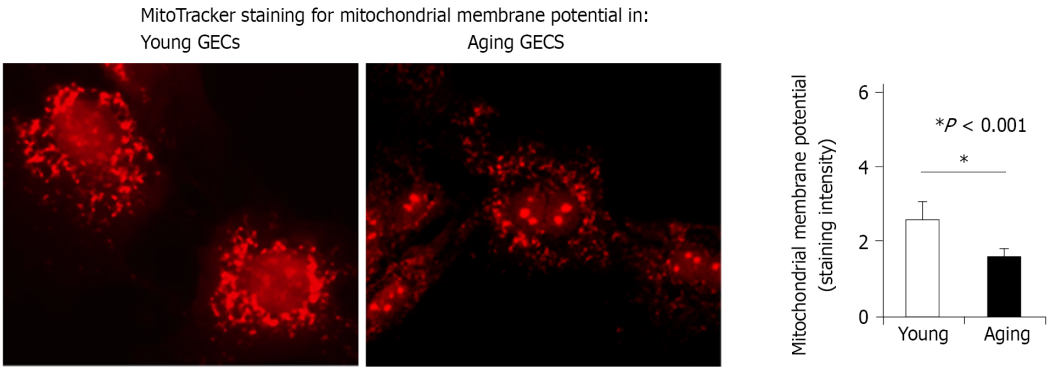

Figure 2 Mitotracker staining for mitochondrial membrane potential in young gastric endothelial cells and aging gastric endothelial cells. Aging gastric endothelial cells (GECs) have significantly reduced mtMP reflecting impaired mitochondrial function *vs* young GECs [reproduced with permission from reference[20], which is an open-access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/)]. GECs: Gastric endothelial cells.

in acute respiratory distress syndrome as well as multiple cardiovascular pathologies [18,27,30,32,64,71,73]. The ubiquitous expression of ACE-2 on ECs in all tissues suggests that SARS-CoV-2 can spread *via* circulation throughout the body and affect multiple organs[55].

The sequential steps of SARS-CoV-2 infection of ECs that result in endothelial pathology and a procoagulant, hypofibrinolytic state of the endothelium are summarized in Figure 3. SARS-CoV-2 utilizes the ACE2 receptors and cellular proteases (TMPRSS-2, CTSB and CTSL) infect the host cells including ECs[51-53,56, 57]. The virus then replicates within the cells and is released into the blood vessels, which then disseminate the virus to distant organs. Severe COVID-19 results in increased production of pro-inflammatory cytokines such as interleukin-6 (IL-6), interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) which is referred to as cytokine storm[29,77,78]. The binding of IL-6 to its receptors on ECs increases vascular permeability, induces capillary leakage, and unleashes a cytokine storm by further increasing the secretion of IL-6, IL-8, and MCP-1 by ECs[29,70,78]. The cytokine storm in COVID-19 patients exposes the endothelium to pro-inflammatory cytokines resulting in leukocyte recruitment and inflammation and can lead to EC death that contributes to increased vascular permeability and end-organ damage[18,29]. In addition, activated ECs produce increased amounts of vWF and factor VIII, which participate in clot formation thereby inducing a pro-coagulant state. Furthermore, ECs produce increased amounts of PAI-1 that inhibits the degradation of clots and induces a hypofibrinolytic state[29,70,78].

The initial SARS-CoV-2 infection and vascular damage in pulmonary tissues can result in the release of ECs into the circulation. Increased numbers of circulating ECs (CECs) have been demonstrated in conditions associated with vascular damage[79-82]. Increased CECs may potentiate the spread to distant extrapulmonary tissues. Numerous extrapulmonary manifestations of SARS-CoV-2 infection such as acute kidney injury, thrombotic complications, myocardial dysfunction and arrhythmia, heart failure, venous thromboembolism, GI symptoms, hepatocellular injury, neurologic illnesses, ocular symptoms, and dermatologic complications have been documented[1]. Endothelial injury may be the underlying mechanism for both pulmonary and extrapulmonary manifestations of COVID-19.

Endothelial cells are critical for vascular regeneration through angiogenesis and vasculogenesis during the injury/lesions healing phase

The process of tissue injury healing involves tissue and vascular regeneration[32,34,75, 83,84]. The latter is mediated by the sprouting of ECs from pre-existing vessels from areas bordering injury (angiogenesis), or the formation of new blood vessels from bone marrow-derived angiogenic precursor cells (vasculogenesis)[22,23,85]. Blood vessel reconstruction is regulated by angiogenic growth factors and involves the activation of genes such as basic fibroblast growth factor (bFGF or FGF-2) and its receptors; VEGF and its receptor; angiopoietins -Ang 1 and Ang 2, and their receptor, COX-2, serum response factor, NGF, stromal-derived factor 1[25]. Our previous studies demonstrated the aging-related decrease in the expression of VEGF and NGF in ECs and that treatment with VEGF and NGF restore angiogenesis in aging gastric ECs (Figure 4)[20, 21]. Furthermore, we showed that local NGF therapy of gastric ulcers increased

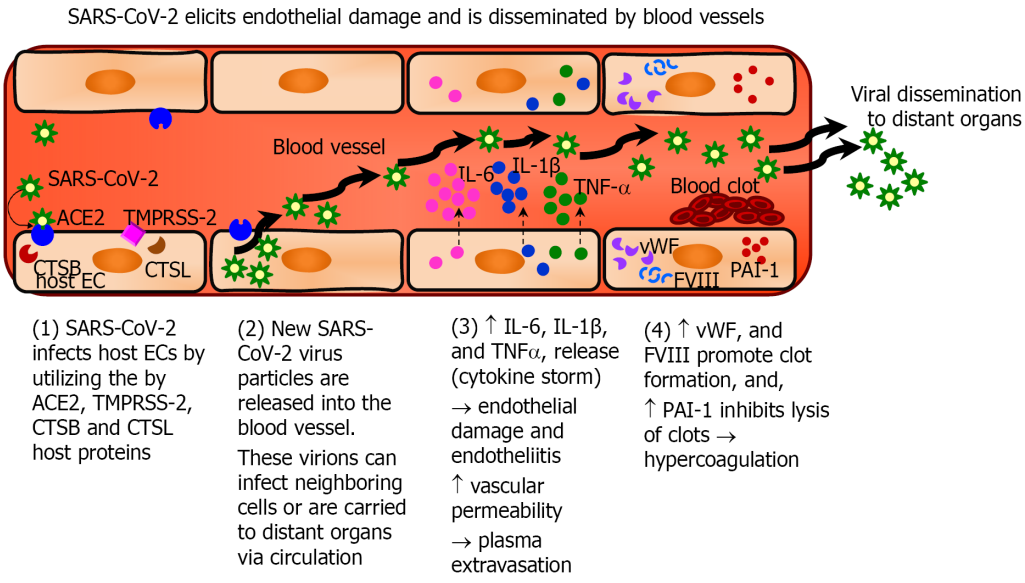

Figure 3 Sequential steps of SARS-CoV-2 infection of endothelial cells and endothelial damage. SARS-CoV-2 infects endothelial cells (ECs) using the host angiotensin-converting enzyme 2 receptors and cellular proteases (transmembrane serine protease 2, and cathepsin B and L). The virus then replicates within the cells and is released into the blood vessels, which then disseminate the virus to distant organs. Severe COVID-19 results in a cytokine storm wherein there is increased production of pro-inflammatory cytokines such as interleukin-6 (IL-6), IL-1, interleukin-1 β (IL-1 β) and tumor necrosis factor-α (TNF-α), and results in endothelial damage and endotheliitis, and demonstrated increased vascular permeability that cause plasma extravasation. Activated ECs produce increased amounts of vWF and factor VIII, and PAI-1, which induce a pro-coagulant, hypofibrinolytic state. SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; ECs: Endothelial cells; ACE2: Angiotensin-converting enzyme 2; TMPRSS-2: Transmembrane serine protease 2; CTSB: Cathepsin B; CTSL: Cathepsin L; IL: Interleukin; TNF-α: Tumor necrosis factor-α.

angiogenesis, promoted revascularization, and accelerated gastric ulcer healing in aging rats[20].

The long-term effects of COVID-19 and its vaccines on endothelium and vasculature remains unknown

SARS-CoV-2 infection was first reported in 2019 and rapid, breakthrough research resulted in the development of several effective COVID-19 vaccines. Although these vaccines have proven effective in reducing the infection and severity of COVID-19, the long-term effects of the disease and the vaccines on ECs and blood vasculature are still to be determined.

POTENTIAL TREATMENTS

Two recent outstanding studies published by the Baishideng Publishing Group in the *World Journal of Virology* outlined the current therapies that have been utilized in COVID-19 treatment[86,87]. We wish to add to this list additional investigational treatments in ongoing clinical trials (Table 3) and describe two additional oral drugs that were announced in early November 2021 as potential COVID-19 treatments Molnupiravir (Merck) and Paxlovid (PF-07321332).

During recent press releases two newest oral drugs inhibiting SARS-CoV-2 replication were recently presented. Are they game changers? On November 4 and 5, 2021 two oral drugs were announced as novel COVID-19 treatments - Molnupiravir (Merck) and Paxlovid (PF-07321332). Both these drugs showed dramatic efficacy in controlling severe COVID-19 infection. The oral drug Molnupiravir (EIDD-2801) was developed by US-based Merck & Co Inc and Ridgeback Biotherapeutics[88] and investigated in a clinical trial (NCT04405570) to eliminate SARS-CoV-2 virus load in infected patients, has since been approved in the UK to treat patients with mild to moderate COVID-19 and at least one risk factor such as older age, diabetes, obesity, and heart disease that predisposes them for developing severe illness. Molnupiravir is the prodrug of the ribonucleoside analog β-D-N4-hydroxycytidine and is rapidly converted by host kinases in plasma to the active 5′-triphosphate form. The latter is a competitive substrate for SARS-CoV-2 RNA-dependent RNA polymerase and causes mutations in the viral genome during replication that makes the virus non-viable. The

Table 3 Summary of the investigational interventions/treatments for COVID-19 in clinical trials

Intervention/ Treatment	Mode of action	Dose	Route	ClinicalTrials.gov Identifier
Ronapreve/REGN-COV2 (REGN10933 and REGN10987)	Monoclonal antibodies against spike proteins	8 g once, or 4 g twice	IV	NCT04425629
Lopinavir/Ritonavir	Inhibitor of the HIV protease and cytochrome P-450 CYP3A	200/ 50 mg; (4 tablets twice a day on day 1 followed by 2 tablets twice a day for 9 d)	Oral	NCT04403100
Remdesivir (RDV, GS-5734, Veklury)	Inhibitor of RNA-dependent RNA polymerase	200 mg on day 1 followed by 100 mg for 4-9 d	IV	NCT04292899
Hyperimmune Plasma (COV19-PLASMA)	Immunotherapy	250-300 mL up to 3 times over 5 d	IV	NCT04321421
Tocilizumab (TCZ, ROACTEMRA)	Humanized anti-IL6 receptor monoclonal antibody	8 mg/kg single infusion, up to 800 mg	IV	NCT04320615
Sarilumab (Kevzara, REGN88, SAR153191)	Monoclonal antibody against IL-6 receptor alpha	200 mg or 400 mg; single dose and multiple doses	IV	NCT04315298
Anakinra (KINERET)	Monoclonal antibody against the IL-1 receptor	100 mg daily up to 28 d	SC	NCT04330638
Siltuximab (SYLVANT)	Chimeric anti-IL-6 antibody	11 mg/kg single infusion	IV	NCT04330638
Eculizumab	Monoclonal antibody against complement protein C5	900 mg every 7 d	IV	NCT04288713
Methyl-prednisolone (MP)	Immunosuppression against cytokine storm	80 mg/kg IV bolus, followed by infusion of 80 mg/d for at least 8 d and then oral MP 16 mg or 20 mg IV twice daily	Oral-IV	NCT04323592
Heparin	Antithrombotic agents	10 units/kg/h	IV	NCT04367831
Enoxaparin (Lovenox)	Antithrombotic agents	1 mg/kg	SC	NCT04367831
Dexamethasone	Immunosuppression against cytokine storm	20 mg/d (5 d) then 10 mg/d (5 d)	IV	NCT04325061
Vitamin C	Antioxidant	12 g infusion twice a day for 7 d	IV	NCT04264533
Melatonin	Antioxidant	3 or 30 mg three times a day for 14 d	Oral	NCT04784754
CoQ10	Antioxidant	500 mg/day for 6 wk	Oral	NCT04960215

IL.: Interleukin.

specific action of this drug on SARS-CoV-2 infection of ECs is not known.

The second drug, Paxlovid (PF-07321332; ritonavir) is a SARS-CoV-2 protease inhibitor antiviral therapy[26]. PF-07321332 is an inhibitor of the SARS-CoV-2 3-chymotrypsin-like cysteine protease that is essential for SARS-CoV-2 replication[26, 89]. Ritonavir is a protease inhibitor that slows down the metabolism/breakdown and therefore, increasing the bioavailability of other protease inhibitors including PF-07321332 in the body[90]. Studies published on November 2, 2021, in Science reported the discovery and characterization of PF-07321332 (Paxlovid)[26]. These studies demonstrated that Paxlovid inhibits SARS-CoV-2 replication *in vitro* in human adenocarcinoma-derived alveolar basal epithelial and differentiated normal human bronchial epithelial cells[26]. This drug showed *in vitro* coronavirus antiviral activity against all coronaviruses infecting humans and excellent off-target selectivity and *in vivo* safety profiles.

That study also showed the efficacy of orally administered 300 or 1000 mg/kg PF-07321332 against SARS-CoV-2 infection *in vivo* in a mouse model challenged intranasally with SARS-CoV-2 MA10 (CCID50). PF-07321332 Limited cellular infiltration by SARS-CoV-2 and protected lung tissue from damage compared to placebo treatment in that study[26]. Most importantly, the interim analysis of the Paxlovid human clinical trial demonstrated a dramatic approximate 90% reduction in COVID-19-related hospitalization or death in high-risk patients treated within 3 to 5 d of symptom onset compared to placebo. Since this drug inhibits virus replication the chance of endothelial infection and dissemination of virus *via* blood vessel is reduced. We postulate that ECs and blood vessels are likely an important part of this drug's clinical efficacy. Naturally, this contention requires further careful analysis and confirmation, and in-depth insight, since the biological effects of these drugs are

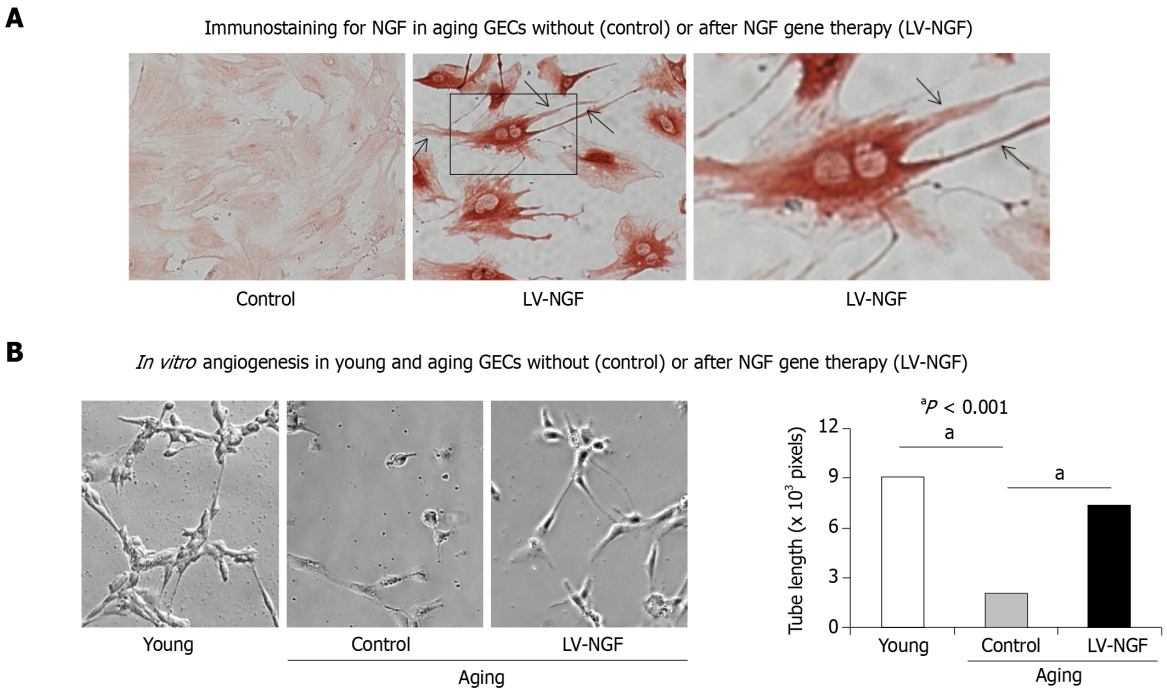

Figure 4 Nerve growth factor gene therapy increases nerve growth factor expression and reverses impaired *in vitro* angiogenesis in aging gastric endothelial cells. A: Nerve growth factor (NGF) gene therapy of aging Gastric endothelial cells (GECs) using lentiviral-NGF (LV-NGF) induced NGF expression (brown staining) and extensive, long filopodia (arrows) reflecting a change in these cells to an angiogenic phenotype; aging GECs without gene therapy (negative controls) have minimal NGF expression and lack filopodia; B: NGF gene therapy with LV-NGF resulted in 3.7-fold increased *in vitro* angiogenesis at 6 h in aging GECs *vs* negative controls (control). Panels are representative images of capillary-like tube formation. Original magnification: × 200. Data are means ± SD (n = 6). ($^aP < 0.001$). NGF: Nerve growth factor; GEC: Gastric endothelial cells; LV: Lentiviral. Reproduced with permission from reference[20], which is an open-access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

largely unknown[91,92]. This sentiment and discussion regarding these oral drugs are summarized in the November 10, 2021 Nature article titled COVID antiviral pills: what scientists still want to know[91]. On December 22, 2021, the US Food and Drug Administration issued an emergency use authorization of Paxlovid to treat mild and moderate COVID-19 (https://www.fda.gov/news-events/press-announcements/coronavirus-covid-19-update-fda-authorizes-first-oral-antiviral-treatment-covid-19).

Other drugs that may be repurposed for COVID-19 treatment include melatonin, coenzyme Q 10 (CoQ10). Melatonin with its anti-inflammatory and anti-oxidative effects can protect against bacterial and viral infections[93-95] and an ongoing clinical study is investigating the efficacy of melatonin in COVID-19 (NCT: 04784754). A clinical trial is investigating the effect of high-dose CoQ10 in long-term COVID-19 patients (NCT: 04960215). The use of growth factors - VEGF, NGF, EGF and KGF, and treatment with adipose-derived stem cells (ADSCs) may be useful for COVID-19 therapy in both the initial and especially the regenerative, healing phase of the disease. A recent study demonstrated that ADSCs release exosomes that secrete various growth factors such as NGF, IGF1, HGF, *etc*.) that may alleviate the cytokine storm in COVID-19 patients[96].

CONCLUSION

While respiratory epithelium is the main route of virus entry, the ECs lining blood vessels are an integral part of COVID-19 disease progression and multi-organ spread. COVID-19 not only affects the lungs and respiratory system but also gastrointestinal tract, liver, pancreas, kidneys, heart, brain, and skin. Blood vessels serve as conduits for the virus dissemination to these distant organs. Importantly, ECs are also critical for vascular regeneration during injury/lesions healing and restoration of vascular network. In the present article, we reviewed the role of the endothelial and vascular components as major targets for COVID-19-induced tissue injury, spreading to various organs, and injury healing, and the current treatments for COVID-19 including the most recent oral drugs Molnupiravir and Paxlovid.

ACKNOWLEDGEMENTS

I dedicate this article to my awesome wife Hella Gergely Tarnawski, who encouraged me to write this article and has been my inspiration. Tarnawski A.S.

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World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2022 January 21; 28(3): 290-309

DOI: 10.3748/wjg.v28.i3.290

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

FRONTIER

Eliminating viral hepatitis in children after liver transplants: How to reach the goal by 2030

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Author contributions: Poovorawan Y substantially contributed to conception of the study and drafted the manuscript; Sintusek P and Thanapirom K wrote the manuscript; Poovorawan Y and Komolmit P made critical revisions related to the intellectual content of the manuscript; all authors read and approved the final version of the manuscript.

Conflict-of-interest statement: The authors declare that they have no conflict of interest.

Supported by The Center of Excellence in Clinical Virology, Faculty of Medicine, King Chulalongkorn Memorial Hospital, Chulalongkorn University, Bangkok 10330, Thailand and Thai Pediatric Gastroenterology, Hepatology and Immunology (TPGHAI) Research Unit, Department of Pediatrics, Faculty of Medicine, King Chulalongkorn Memorial Hospital, Thai Red Cross, Chulalongkorn University, Bangkok 10330, Thailand.

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Abstract

Viral hepatitis infections are a great burden in children who have received liver transplant. Hepatotropic viruses can cause liver inflammation that can develop into liver graft fibrosis and cirrhosis over the long term. Immunological reactions due to viral hepatitis infections are associated with or can mimic graft rejection, rendering the condition difficult to manage. Prevention strategies using vaccinations are agreeable to patients, safe, cost-effective and practical. Hence, strategies to eliminate viral hepatitis A and B focus mainly on immunization programmes for children who have received a liver transplant. Although a vaccine has been developed to prevent hepatitis C and E viruses, its use is not licensed worldwide. Consequently, eliminating hepatitis C and E viruses mainly involves early detection in children with suspected cases and effective treatment with antiviral therapy. Good hygiene and sanitation are also important to prevent hepatitis A and E infections. Donor blood products and liver grafts should be screened for hepatitis B, C and E in children who are undergoing liver transplantation. Future research on early detection of viral hepatitis infections should include molecular techniques for detecting hepatitis B and E. Moreover, novel antiviral drugs for eradicating viral hepatitis that are highly effective and safe are needed for

Specialty type: Gastroenterology and hepatology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Received: June 3, 2021
Peer-review started: June 3, 2021
First decision: July 27, 2021
Revised: August 12, 2021
Accepted: January 6, 2022
Article in press: January 6, 2022
Published online: January 21, 2022

P-Reviewer: Elshimi E
S-Editor: Wu YXJ
L-Editor: A
P-Editor: Wu YXJ

children who have undergone liver transplantation.

Key Words: Viral hepatitis; Children; Adolescent; Liver transplantation; Infection; Elimination

Core Tip: Viral hepatitis infections are a great burden for pediatric liver transplant recipients. Strategies to prevent infection include immunization, good sanitation and screening donor blood products and liver grafts for hepatitis B, C and E. In children infected with viral hepatitis who have received a liver transplant, early detection is crucial to guide proper management, as the infection can mimic or cause graft rejection. Effective antiviral therapy should be initiated when treating children with hepatitis B and C. Patients infected with hepatitis B who have undergone successful viral eradication should be revaccinated to maintain high hepatitis B surface antibodies to guarantee immunoprotection.

Citation: Sintusek P, Thanapirom K, Komolmit P, Poovorawan Y. Eliminating viral hepatitis in children after liver transplants: How to reach the goal by 2030. *World J Gastroenterol* 2022; 28(3): 290-309
URL: https://www.wjgnet.com/1007-9327/full/v28/i3/290.htm
DOI: https://dx.doi.org/10.3748/wjg.v28.i3.290

INTRODUCTION

Viral hepatitis is an infectious disease leading to high morbidity and mortality, especially in endemic areas such as Asia. Hepatitis viruses are hepatotropic and are classified into types A, B, C, D and E. In immunocompromised patients, including children who have undergone liver transplantation (LT) and typically receive lifelong immunosuppressants, nearly all viral hepatitis infections are chronic, progressing to liver fibrosis and cirrhosis in the long term. Hepatitis A is the only hepatitis virus that presents as an acute self-limiting infection but that is more severe in immunocompromised patients than in healthy individuals. Because viral hepatitis places a heavy burden on patients, strategies for prevention, early detection and prompt, effective management are crucial for graft survival and long-term outcomes in children after LT. In this review, we focus on lessons learned and future opportunities to develop effective strategies to eliminate hepatitis A, B, C and E in children after LT.

BIOGRAPHY

Yong Poovorawan (Figure 1), MD is currently the Professor and the head of the Center of Excellence in Clinical Virology at the Faculty of Medicine, Chulalongkorn University, Bangkok. Professor Poovorawan obtained the medical degree in 1974 and his specialization in paediatrics in 1978 from King Chulalongkorn Memorial Hospital, Chulalongkorn University. In 1984, he became a research fellow on the field of paediatric hepatology at King's College Hospital Medical School, London. Professor Poovorawan has been working in the Department of Pediatrics at Chulalongkorn University, beginning as a lecturer and becoming Professor in 1991. Professor Poovorawan has received many research awards and honours, including the Outstanding Researcher Award in 1997 from the National Research Council of Thailand, Outstanding Scientist Award in 1997 from the Foundation for the Promotion of Science and Technology under the Patronage of His Majesty the King, Mahidol University-B-Braun Award in 2002, Thailand Research Fund Award in 2004 and has been nominated Senior Research Scholar by the Thailand Research Fund since 1997. He also received the Outstanding Best Teachers Award in 2004 from the Thailand National University Teacher Association. He is a leader who has been working on Viral hepatitis in Thailand. Outstanding Prof. Thailand Research Fund (2012-2014), Research Chair Grant, NSTDA (2014), Outstanding Achievement Doctor from the

Figure 1 Yong Poovorawan, MD, Professor, Excellence Center of Clinical Virology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

Medical Council of Thailand (2018), Achievement Award in Virology, Genetics Society of Thailand (2018), Achievement Award from the National Vaccine Institute of Thailand (2019). His work on avian influenza in Thailand also received outstanding research awards from the Thailand Research Fund in 2004 and the National Research Council in 2006. He is a member of the expanded programme on immunization vaccine, viral hepatitis and emerging diseases of the Center Disease Control, Ministry of Public Health. Professor Poovorawan has authored and co-authored more than 614 publications in the fields of hepatitis, paediatric hepatology and virology, with H-index 66 on Google Scholar.

HEPATITIS A VIRUS

Manifestations of hepatitis A virus (HAV) infection mainly derive from the immunologic responses to the virus and might present as severe acute liver failure in healthy children[1]. The incidence of fulminant hepatic failure from HAV infection varies from 0.1% to 1% but increases in patients with chronic liver disease[2-4]. HAV is rarely reported in children after LT. In our centre, no children have been admitted with HAV after LT[5], likely because Thailand has significantly decreased the HAV infection rate in past decades[6]. However, HAV can mimic graft rejection or presents as recurrent HAV post-LT[7]. Immunization is the mainstay of prevention and should be given before LT. Short- and long-term studies (ranging from 2-48 mo) of immunologic responses to HAV vaccines in adults post-LT have revealed variable seroprotection rates ranging from 26%-97% after 2 doses of the vaccine[8-11]. In a 4-wk assessment, Ferreira *et al*[12] compared HAV vaccine immunogenicity between children with chronic liver disease and healthy controls and found that 97% of the former and 100% of the latter showed seroconversion, with geometric mean titres of 812.4 mIU/mL and 2344.90 mIU/mL, respectively, after 2 doses of the HAV vaccine[12]. However, no study has reported HAV vaccine immunogenicity in children post-LT. Arslan *et al*[13] found that 18% and 29% of adult patients lost humoral immunity to HAV at 1 and 2 years, respectively, post-LT. Interestingly, one case study reported a 55-year-old man who was previously immunized and was HAV-IgG-positive but had an acute HAV infection in-hospital post-LT[14]. This case report suggested that rapid HAV seroconversion after LT should be regularly monitored and that revaccination should be considered for patients with a loss of HAV immunity. We recommend providing HAV vaccines to all children waiting for LT, as the humoral immune response to the HAV vaccine is favourable[12]. Nevertheless, HAV vaccines are provided only for children older than 1 year, and younger children may require LT before being eligible for the HAV vaccine. Hence, post-LT HAV immunization is needed. Further studies should be conducted regarding long-term immunologic responses of HAV to confirm the efficacy of 2-dose HAV vaccines in immunocompromised children. Apart from the vaccine, other necessary strategies include improving sanitation and avoiding uncooked food.

HEPATITIS B VIRUS

Immunization: A public health weapon for preventing hepatitis B virus infection

Pre-LT: Since universal hepatitis B virus (HBV) vaccination programmes began in the 1990s[15], HBV infection prevalence has rapidly decreased worldwide. HBV vaccine series that include vaccines at birth, 1-2 and 6-12 mo can reduce mother-to-child transmission, the major mode of HBV transmission in children, from 65%-90% to 3.6%-4.0%[16,17]. Indeed, the seroprotective rate after a complete HBV series is > 95%[18]. In our cohort study, although seroprotective rates decreased to 44% over a 20-year follow-up, 93.1% of the children exhibited seroconversion after a booster dose[19]. The presence of immune memory cells after the booster dose confirmed waning immunity with an anamnestic response, indicating increased levels of hepatitis B surface antibodies (anti-HBs)[19]. However, in immunocompromised patients with chronic liver diseases or cirrhosis, revaccination yields unsatisfactory outcomes, with seroconversion rates of 37.0%-90.9% on conventional schedules[20-28] and 16%-72% on accelerated/super-accelerated[29-37] schedules. Many studies on HBV schedules have been conducted to improve immunologic responses after revaccination in nonresponders, mainly using adult data, with different doses, routes, vaccine types, numbers and injection intervals. Regardless, no differences in the efficacy of these regimens have been shown[38]. Overall, time is a concern for participants awaiting LT, and super-accelerated or accelerated regimens should be considered for short-term prevention of HBV infections during and after LT[39].

During LT: External sources of HBV transmission, such as blood products, medical instruments and transmission by hospital personnel or close contacts, are concerns. Anti-HBs may decline after excessive plasma loss during surgery, and occult HBV infections from positive hepatitis B core antibody (anti-HBc) blood products have been reported[40].

Post-LT: Immunologic loss of HBV is common after LT [41], and *de novo* hepatitis B infection (DNH) was observed in our paediatric LT centre[42]. DNH is likely related to acquired HBV infections from endemic environments or from HBV reactivation from positive anti-HBc allografts during immunologic loss[43-47]. In our centre, the anti-HBs loss rate increased rapidly after LT, and 46%, 57% and 82% of patients had anti-HBs levels of < 10 mIU/mL at 1 year, 2 years and > 3 years after LT, respectively. One case of DNH was detected at 3 years after LT, though anti-HBs levels were > 1000 mIU/mL before LT[42]. Hence, regular monitoring for anti-HBs and revaccination after LT are crucial. Studies of immunogenicity to HBV revaccination after LT have reported higher humoral immune responses in children than in adults (up to 100% *vs* 33.3%-63.8%); however, immunity waned, and the patients needed frequent booster doses to maintain high seroprotective levels[43]. In healthy adults not responding to conventional vaccine schedules, a systematic review found no differences in seroconversion rates according to dosage or vaccine administration route[38]. However, to date, no study has been conducted involving children in this population. We conducted studies of immunologic responses to standard *vs* double-dose HBV vaccine series (at 0, 1 and 6 mo) in children after LT exhibiting anti-HBs loss and found response rates of 91.6% and 85% after a 6-mo follow-up, with no statistically significant difference in anti-HBs level between the two regimens (unpublished data). Hence, short-term assessment revealed that HBV revaccinations in children after LT are highly effective and safe.

Positive anti-HBc allografts are considered a major risk factor of DNH after LT, especially in patients without prior seroprotection or rapid anti-HBs loss after LT. In addition to being revaccinated 3-6 mo after LT, other strategies to prevent DNH include antiviral therapy and/or passive immunity with hepatitis B immunoglobulin (HBIG). Unlike the many studies that have used adult data and investigated several strategies, few studies of prophylactic strategies against DNH have been conducted in children who receive positive anti-HBc allografts[43,44,48-51]. Song *et al*[48] reported the efficacy of pre- and post-LT HBV vaccinations to prevent DNH and recommended a prophylactic strategy to maintain anti-HBs levels at ≥ 1000 mIU/mL pre-LT and ≥ 200 mIU/mL post-LT without antiviral consideration. The DNH rate when using this strategy was 1.3%[48]. However, anti-HBs levels may rapidly decline after LT owing to the massive immunosuppression involved. In such cases, antiviral therapy should be added in parallel until the appropriate revaccination time after LT (usually 3-6 mo) and until anti-HBs levels increase to ≥ 200 mIU/mL after revaccination.

Children with chronic HBV infections are rarely indicated for LT because they are usually asymptomatic in the stage of hepatitis B e-antigen (HBeAg)-positive chronic

infection or HBeAg-positive chronic hepatitis. Thus far, immunoprophylaxis data on recurrent HBV infections after LT are mainly based on adult data.

In summary, strategies to prevent HBV infection before, during and after LT mainly include active immunization. Super-accelerated and accelerated vaccines may be considered for timely protection prior to LT (to keep anti-HBs levels ≥ 1000 mIU/mL if possible). However, in children, anti-HBs levels should be regularly monitored, and revaccinations should be provided to maintain high anti-HBs levels (≥ 200 mIU/mL).

The future of HBV elimination after LT

Despite antiviral HBIG and active HBV immunization strategies, DNH has been reported in 0.9%-4.0% of both paediatric and adult LT patients[48,52,53]. Table 1 summarizes the risk factors for DNH. An escape mutation in the "a" determinant region within the hepatitis B surface antigen (HBsAg) that develops before LT, after HBV vaccination, or after HBIG administration post-LT should be considered[54]. In this situation, antiviral agents play a major role in preventing DNH, and long-term assessment for drug resistance should be considered. We recommend including pre-LT evaluations for HBV by serological, molecular and virological methods. Liver donors and allografts should be evaluated for covalently closed circular DNA (cccDNA) and HBV viral loads in cases of suspected occult infection with an escape mutant.

How to treat de novo hepatitis B infection in children after LT

Su *et al*[54] found that after DNH occurred in children post-LT, more than half (5/9) exhibit seroconversion after lamivudine therapy. However, one child carried a tyrosine-methionine-aspartic acid-aspartic acid (YMDD) motif mutation, and the authors switched antiviral agent from lamivudine to adefovir dipivoxil. To date, no consensus treatment for DNH has been reached[43,54-56]. Antiviral therapy for DNH might follow the guidelines for treating HBV infections in children (Table 2). In our unit, one patient with DNH was treated with interferon-α for 1 year without a response, even though this child exhibited HBsAg seroconversion after 6 mo of entecavir therapy. We revaccinated him against HBV after HBsAg clearance following entecavir therapy. This child received an HBV revaccination series (0, 1 and 6 mo) and maintained anti-HB levels of > 1000 mIU/mL without a rebooster at a 44-mo follow-up. Further study on the efficacy of antiviral therapy for DNH and other novel antiviral therapies with less drug resistance and high efficacy in children with DNH should be conducted to determine the best endpoints of HBsAg clearance and anti-HBs appearance. Tenofovir alafenamide (TAF) is a novel tenofovir product with improved properties for avoiding kidney and bone-related adverse events due to tenofovir disoproxil fumarate (TDF). Compared with TDF, TAF has non-inferior efficacy and a good safety profile[57]. Nevertheless, data for post-transplant adults and children receiving TAF are lacking. Only a small single-centre study of adult liver-transplant recipients found that TAF (25 mg/d) displayed high antiviral efficacy in preventing HBV recurrence without affecting immunosuppressive medications or graft functioning and had a good safety profile[58]. TAF is a promising antiviral therapy for adolescents diagnosed with DNH.

Other novel antiviral therapy

As mentioned, current HBV prophylaxis and therapies do not completely eradicate HBV infections in most cases, requiring lifelong medication. Thus, effective and finite HBV treatment remains an unmet medical need, and new therapeutic approaches and drugs are necessary to achieve a functional cure (mainly defined as a loss of when HBsAgs off therapy). Multiple novel drugs targeting different steps in the HBV life cycle are being developed. Antiviral and host-targeting agents are the two main drugs being studied. The major HBV-target-specific categories of antiviral drugs are hepatocyte-entry receptor inhibitors (*e.g.*, bulevirtide, formerly myrcludex B)[59,60], cccDNA inhibitors, nucleocapsid-assembly modulators (core protein allosteric modulators, *e.g.*, JNE-56136379)[61], post-transcriptional control inhibitors (RNA interference drugs, *e.g.*, ARC-520)[62], HBsAg-release inhibitors (nucleic-acid polymers, *e.g.*, REP 2139 and 2165)[63] and HBV DNA polymerase inhibitors. Therapies that target host immune responses include Toll-like receptor (TLR)-7 (*e.g.*, GS-9620, vesatolimod)[64], TLR-8 (GS-9688, selgantolimod)[65], and TLR-9 agonists, checkpoint inhibitors (anti-programmed death 1 and anti-programmed death-ligand 1)[66] and therapeutic vaccines[67]. These drugs are currently in phase I and II clinical trials that mainly include non-transplant adult patients and indicate a promising future for HBV eradication. No data are available on the efficacy of these new drugs

Table 1 Risk factors of *de novo* hepatitis B infection in children after liver transplantation

Risk factors
Positive anti-HBc donor[40]
Positive-intrahepatic HBV DNA[40]
Liver graft HBV DNA > 1000 copies[40]
Intraoperative fresh-frozen plasma transfusion > 400 mL[40]
Positive-anti-HBc recipients[40]
Pre-operative anti-HBs < 1000 mIU/mL[40,43,48]
Post-operative anti-HBs < 100-200 mIU/mL[48,53]
Hepatitis B surface mutation (within the "a" determinant region[54])

Anti-HBc: Hepatitis B core antibody; anti-HBs: Hepatitis B surface antibody.

Table 2 Antiviral agents for hepatitis B infection in children[44]

Medication	Licensing	Dose and duration	HBsAg loss (%)	Resistance (%)
IFN-α-2b	≥ 1 yr	6 million IU/m^2 three times weekly for 6 mo	1-2	0
Lamivudine	≥ 2 yr	3 mg/kg daily for ≥ 1 yr	0	19-64
Entecavir	≥ 2 yr	0.25-0.5 mg daily for ≥ 1 yr	0.52	0.7-1.2
Tenofovir dipovaxil fumarate	≥ 12 yr	300 mg daily for ≥ 1 yr	0.02	0
Adefovir	≥ 12 yr	10 mg daily for ≥ 1 yr	0	0.9-20

HBsAg: Hepatitis B surface antigen.

against HBV recurrence or *de novo* infection in children after LT. Further studies are needed to determine the impact of the new drugs on these patient groups.

Based on current knowledge of the human immunodeficiency virus (HIV) and hepatitis C virus (HCV), immunomodulators and combination treatments targeting several steps in HBV replication will likely be required to achieve a functional cure for HBV. Preclinical studies are applying this strategy in animal models[68], and clinical trials are investigating combinations of several antiviral drugs or immune boosters with antiviral agents. This new approach using combination therapies will need to be individualized, but many patients may be eligible.

In summary, strategies to eliminate HBV in paediatric liver transplant recipients include HBV immunization both pre- and post-LT. Early detection of HBV infections, especially of escape mutants, which lead to vaccine failure in recipients, and of cccDNA in the livers of positive anti-HBc donors, should be evaluated *via* molecular and viral genetic analysis in the liver tissues of both the donors and recipients. Patients with vaccine failure or DNH should promptly undergo antiviral therapy. Figure 2 shows the proposed strategies to eliminate HBV in children post-LT.

HEPATITIS C VIRUS

HCV infections are a global health problem, with an estimated 71 million people being chronically infected in 2016 and 400000 deaths annually worldwide[69]. Therefore, in 2016, the World Health Organization (WHO) set the goal of eliminating HCV by 2030. There has been significant progress towards this goal in screening policies, improving access to care, and reducing the costs of direct-acting antivirals (DAAs). Compared with adult patients, little attention has been paid to diagnosis, therapy, and prevention for children and adolescents. One reason is that prior to 2017, no DAAs were licensed for use in patients under 18 years old, and evidence was lacking to support paediatric management guidelines and policies. The majority of national HCV policies do not include explicit recommendations for HCV testing and treatment in children and

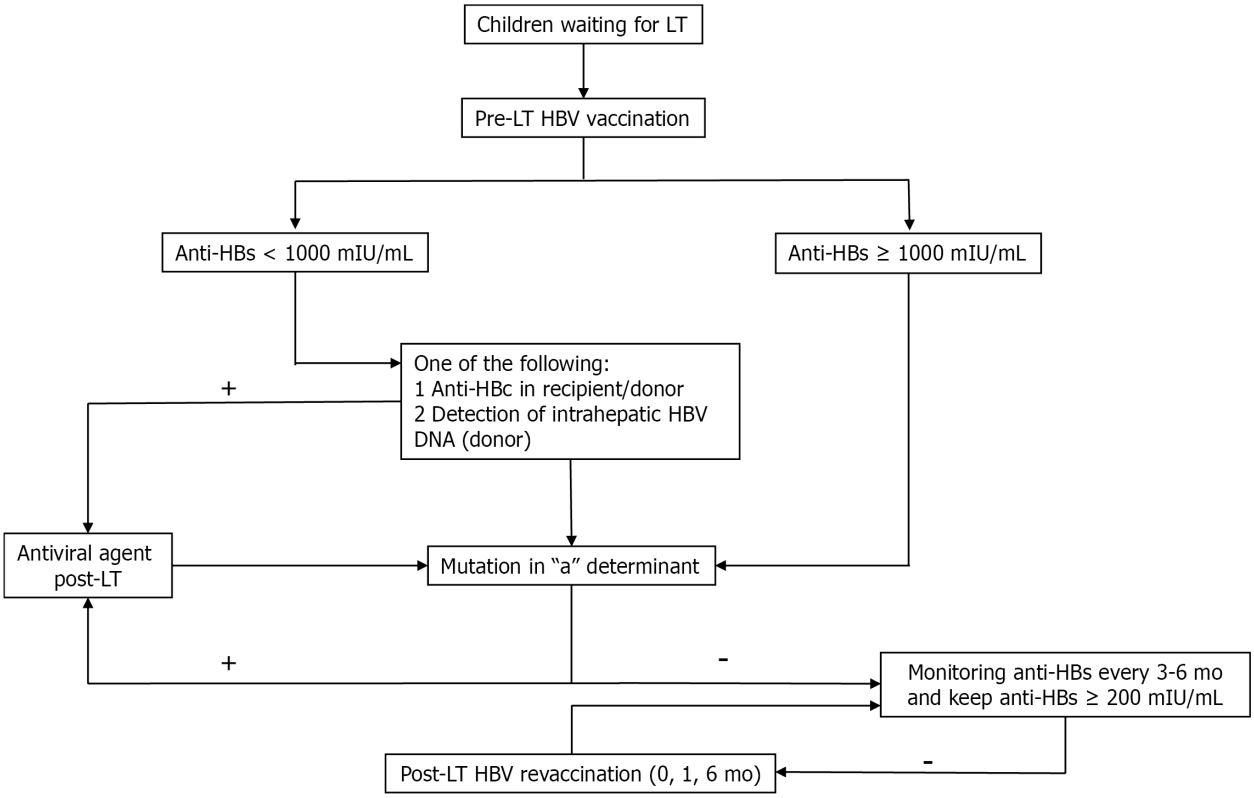

Figure 2 Proposed strategies to prevent *de novo* hepatitis B infection[41-48,52,53]. LT: Liver transplantation; HBV: Hepatitis B virus; anti-HBc: Hepatitis B core antibody; anti-HBs: Hepatitis B surface antibody.

adolescents[70]

Transmission route and natural history

In 2018, the global prevalence of HCV viraemia in populations under 18 years old was 0.13%, with an overall burden of 3.3 million cases[71]. The true HCV infection prevalence in paediatric populations is unknown due to a lack of universal screening strategies. Perinatal transmission is a major cause of recognized HCV infections in children, with transmission rates of 5% from HCV-infected mothers and 10% from HCV-HIV-coinfected mothers[72,73]. Moreover, the opioid epidemic is associated with an expanding ongoing risk of HCV transmission from mothers to children[74]. In the United States, nearly 29000 HCV-infected women gave birth annually from 2011-2014 [75]. Moreover, the transmission risk increases with higher maternal HCV viral loads, HIV coinfections, longer labour durations, amniocentesis or foetal-scalp monitoring, and prolonged membrane rupture[72,76-78]. Several studies from developed countries have reported increased injection drug use as a risk factor of HCV and HIV infections among adolescents[79,80]. Sexual transmission of HCV is also a major factor in men who have sex with men, including those infected with HIV or those who have received a pre-exposure prophylaxis for HIV[81,82].

After vertical HCV transmission, 25%-40% of patients spontaneously clear the infection within the first 4 years of life[83]. Approximately half of infants born with HCV will develop chronic disease that may lead to cirrhosis and hepatocellular carcinoma in late childhood[84]. The natural history of paediatric HCV differs from that of HCV acquired in adulthood. Host factors (*e.g.*, rs12979860 mutation in the *IL28B* gene[85], natural killer cell cytolytic functions[86]) and viral factors (*e.g.*, HCV genotype)[87] are associated with spontaneous clearance of HCV infections. Children with chronic HCV infections are mostly asymptomatic, with mild degrees of hepatitis and fibrosis during childhood and higher rates of spontaneous HCV clearance. Therefore, it is uncommon for children and adolescents to develop HCV-associated end-stage liver disease or hepatocellular carcinoma (HCC)[88]. Comorbidities, including haematological disease with iron overload, obesity, alcohol use, and concomitant viral infections (*e.g.*, HBV or HCV), are associated with accelerated liver fibrosis and cirrhosis development[89]. HCV-related extrahepatic manifestations are less common in paediatric patients than in adult patients[90]. In general, HCV

infections in children and adolescents are related to poor life quality and reduced cognitive functioning[84].

Diagnostic testing

Several current international guidelines recommend anti-HCV testing (with a confirmatory nucleic acid assay for a positive result) for all pregnant women, especially those in high-risk groups, including those with past or current injection drug use, incarceration history, unregulated tattoos/piercings, receipt of contaminated blood products, or exposure in HCV-endemic areas[91-93]. HCV RNA can be found in breast milk and colostrum, but breastfeeding does not increase HCV transmission rates except in HCV-HIV coinfected mothers[94]. All children born to HCV-infected mothers should be tested for HCV infection before 18 mo of age. Because anti-HCV antibodies passed from mothers can persist until 18 mo of age, HCV infection in children younger than 18 mo can be diagnosed by detecting HCV RNA. High-risk adolescents, including those who with histories of injection drug use and men who have sex with men, should be tested for HCV infection[95].

The asymptomatic nature of HCV infection and the high cost of diagnostic screening are the important barriers to detecting and treating HCV-infected patients [96]. Thus, a simple, cost-effective diagnostic method for routine HCV screening especially for low- to middle- income countries is needed. The core antigen of HCV (HCV Ag) is an alternative for screening and diagnosis. This test can be used as a supplemental marker after anti-HCV testing to reduce the requirement of further confirmatory HCV RNA assays[97]. Point-of-care tests of viraemia are related with improvement in access to testing [98].

Treatment DAAs in HCV infection before/after LT

Advancement of oral DAA therapies has resulted in a paradigm shift in treating HCV, with cure rates of > 90% and few adverse effects. DAAs with pan genotypic activity are recommended as preferred regimens for all treatment-naïve and treatment-experienced HCV patients, regardless of age, sex, stage of liver fibrosis, or HIV coinfection[93,99,100]. Conversely, pegylated-interferon-based regimens are no longer recommended. DAA treatment with an approved regimen is recommended for all children and adolescents ≥ 3 years old with HCV infection, regardless of disease severity[101,102]. Early antiviral treatment should be administered to reduce morbidity and mortality if extrahepatic manifestations occur (*e.g.*, glomerulonephritis and cryoglobulinemia).

Adolescents aged 12-17 years who are treatment-naïve or -experienced, without cirrhosis or with compensated cirrhosis (Child-Pugh A) should be treated according to the recommendations for adult patients. For pangenotypic HCV, two DAA regimens are recommended: sofosbuvir (400 mg)/velpatasvir (100 mg) once daily for 8-12 wk, achieving a 95% sustained virological response (SVR)-12 rate (97/102; 1 virological failure) with mild-to-moderate adverse events[103]; a fixed-dose combination of glecaprevir (300 mg)/pibrentasvir (120 mg) once daily for 8 wk, achieving a 100% SVR-12 rate with a good safety profile[104]. Although the clinical trial for glecaprevir /pibrentasvir included only adolescents with HCV genotypes 1-4, this drug was approved by the Food and Drug Administration (FDA) for adults with all genotypes. In 2019, the FDA approved treating genotype-specific HCV with sofosbuvir (400 mg)/ledipasvir (90 mg) for 12 wk in adolescents aged 12-17 years or weighing at least 35 kg with genotypes 1, 4, 5, or 6, without cirrhosis or with compensated cirrhosis[105, 106].

Children aged 3-11 years who are treatment-naïve or treatment-experienced, without cirrhosis or with compensated cirrhosis (Child-Pugh A) with any HCV genotypes should be treated with the FDA-approved regimens of a fixed-dose combination of sofosbuvir (200 mg)/velpatasvir (50 mg) for those aged > 6 years weighing ≥ 17 kg and sofosbuvir (150 mg)/velpatasvir (37.5 mg) for 12 wk[103] in those with weighing < 17 kg. One trial found that for children aged 3-11 years, the fixed-dose combination of glecaprevir (250 mg)/pibrentasvir (100 mg) for those weighing 30-44 kg, glecaprevir (200 mg)/pibrentasvir (80 mg) for those weighing 20-29 kg, and glecaprevir (150 mg)/pibrentasvir (60 mg) for those weighing 12-19 kg for 8-16 wk achieved a 96% SVR-12 rate, without drug-related severe adverse events[107]. However, this formulation is not yet FDA approved.

Overall, DAA-experienced children and adolescent patients with HCV are rare in clinical practice (Table 3). Because data for these populations are limited, DAA-experienced paediatric patients with HCV infections should be treated using the guidelines for adult patients.

Table 3 Recommended direct-acting antiviral regimens for children who are naïve to or experienced with direct-acting antiviral therapy [101,102]

Age	Genotype	No cirrhosis/ cirrhosis	Recommended regimens of DAAs	Duration (wk)
12-17 yr	Pan-genotypes	No cirrhosis	Sofosbuvir 400 mg/ velpatasvir 100 mg	12
		Compensated cirrhosis (Child-Pugh A)	Glecaprevir 300 mg/pibrentasvir 120 mg	8-12
12-17 yr or BW ≥ 35 kg	1, 4, 5, 6	No cirrhosis	Sofosbuvir 400 mg/ledipasvir 90 mg	12
		Compensated cirrhosis (Child-Pugh A)	Sofosbuvir 200 mg/velpatasvir 50 mg (BW ≥ 17 kg)	
3-11 yr	Pan-genotypes	No cirrhosis	Sofosbuvir 150 mg/velpatasvir 37.5 mg (BW < 17 kg)	12
		Compensated cirrhosis (Child-Pugh A)	Glecaprevir 250 mg/pibrentasvir 100 mg (BW 30-44 kg); Glecaprevir 200 mg/pibrentasvir 80 mg (BW 20-29 kg); Glecaprevir 150 mg/pibrentasvir 60 mg (BW 12-19 kg)	12; 8-16; 8-16; 8-16;

BW: Body weight.

LT in paediatric patients in the DAA era

Children and adolescents with chronic HCV infections rarely require LT for complications from liver cirrhosis or HCC; recurrent HCV after LT is also clinically rare. In a retrospective study of the United Network of Organ Sharing database, Gupta *et al*[108] found that 120 paediatric patients received transplants for chronic HCV infections in 1994-2010. One-year and 3-year survival rates were 97% and 89%, respectively, in patients with post-paediatric end-stage liver diseases. Pre-LT recipient factors, good surgical technique, and effective treatment for HCV infections are associated with good prognostic outcomes in paediatric patients after LT[108]. Patients who achieve an SVR have less mortality than do those without SVR after treatment. Treatment has better effects on disease outcomes if it is started before cirrhosis[109]. To prevent long-term liver disease and HCV spread, antiviral therapy should be available in childhood.

LT for HCV-related diseases has decreased in the era of DAA treatment. Treating patients before LT reduces the chance of graft dysfunction after LT and may stabilize or improve liver function. SVR before LT may lead to the delisting of some patients [110]. Patients with decompensated (Child-Pugh B or C) cirrhosis who have model for end-stage liver disease (MELD) scores of < 20 without HCC and are awaiting LT should be treated with DAAs before LT. The recommended regimen is sofosbuvir (400 mg)/velpatasvir (100 mg) plus weight-based ribavirin 1000-1200 mg/day for 12 wk or sofosbuvir (400 mg)/velpatasvir (100 mg) for 24 wk in those with contraindications for ribavirin. Patients with MELD scores > 20 should undergo transplantation first and treated for HCV infection after LT if the waiting time is < 6 mo[111,112].

Future treatment

Vaccines: Despite the high curative rate of HCV infections by DAAs, high-risk populations remain at risk of reinfection, even after successful treatment. Preventing new HCV infections is vital and may result in the WHO's 2030 global elimination goal. A prophylactic HCV vaccine might also help to achieve this goal by preventing transmission. Nevertheless, no vaccine for preventing HCV infections has been approved to date.

A recent phase 1-2, randomized, double-blind, placebo-controlled trial by Page *et al* [113] enrolled adults aged 18-45 years who had injected drugs within 90 d. These adults received either an intramuscular injection of a recombinant chimpanzee adenovirus type-3 vector-priming vaccination (ChAd3-NSmut vaccine) on day 0 and a recombinant modified vaccine (Ankara, MVA-NSmut vaccine) booster on day 56 (vaccine group) or a saline placebo on days 0 and 56. Despite inducing HCV-specific T-cell responses and lowering peak HCV RNA levels, the vaccine failed to prevent chronic HCV infection compared with placebo[113]. The innate variability of HCV enveloped proteins and the limited knowledge of HCV protein structures are barriers to developing an HCV vaccine. Future work should determine the optimal HCV epitopes to target vaccine development.

HEPATITIS E VIRUS

Hepatitis E virus (HEV) was first discovered in the 1980s and normally manifests as an acute self-limited condition[114], though chronic HEV infection courses were recognized in 2008 in organ-transplant recipients[115]. HEV infection seroprevalence varies from 0.3%-75.6% depending on the area and diagnostic method[116-121]. HEV is transmitted mainly *via* the faecal-oral route, but mother-to-child[122], liver graft-to-recipient and plasma-derived-product transmission[123,124] have been reported. One study reported HEV transmission *via* liver graft[125], and several cases of HEV infections transmitted by blood transfusion have been reported. These findings have led to universal HEV-RNA testing in blood donors in many countries[123,124,126,127]. To detect HEV infection in immunocompetent children, primary testing with anti-HEV IgG and IgM is reasonable, and HEV RNA in stool and serum samples should be assessed in highly suspected cases that yielded negative results by serological methods[128]. However, serum HEV RNA analysis is preferable in immunocompromised patients, as they cannot mount an antibody response[129]. HEV infection has clinical impacts in immunocompromised hosts, especially in those needing organ transplantation. Moreover, HEV infections can be asymptomatic pre-existing chronic liver diseases or solid organ transplantation[130-133], or liver-associated morbidity due to progressive fibrosis and cirrhosis may be present[134]. Additionally, these conditions increase the risk of graft rejection[132]. In 2014, the European Association for the Study of the Liver proposed a well-organized stepwise plan for managing HEV infections in both adults and children after organ transplantation[128]. Once HEV infection is detected in children after LT, immunosuppression should be reduced when possible, and these children should be followed up within 3 mo. HEV-RNA clearance may occur in one-third of these patients. If chronic HEV infection persists, antiviral therapy with ribavirin (15 mg/kg/d) should be administered for at least 3 mo[135], and HEV clearance should be monitored monthly *via* PCR. Three promising recombinant vaccines against HEV with high efficacy have been developed[136] and can maintain seroprotection for > 4.5 years[137,138]. Many studies and case reports of HEV-infected children after LT have resulted from this increased awareness. Table 4 shows the results of previous studies[130-133,139,140] and HEV infection data for children after LT in our centre (unpublished data).

Strategies to eliminate HEV infection include prevention by implementing hygienic measures and thoroughly cooking food, screening plasma-derived products from immunocompromised patients and, developing an HEV vaccine. Early detection and effective treatment with antiviral agents in infected patients are also crucial.

Future: How to eliminate HEV after LT

Diagnostic testing for HEV infection: As chronic HEV infections in children after LT lead to progressive hepatitis and liver fibrosis, suspected cases should be tested. As serologic testing is insufficient to detect HEV infections in immunosuppressed patients, HEV infections should be diagnosed based on HEV-RNA detection in specimens. Protzer *et al*[141] reported molecular detection of HEV in liver-biopsied tissues from four liver-transplanted patients, whereas serology only detected two (Mikrogen assay). Prost *et al*[142] compared HEV-RNA detection in clinical liver-biopsy tissues between *in situ* testing and qPCR from paraffin-embedded liver tissues and found that qPCR was more effective. Additionally, Ankavay *et al*[143] found that detecting the open reading frame 2 (ORF2) protein of HEV *via* immunohistochemistry of liver tissues can be used as a rapid histopathological method to diagnose HEV infections. The sensitivity and specificity of this technique were the same as those of tissue PCR for HEV RNA. The ORF2 clone 1E6 antibody yielded the highest diagnostic accuracy and was more sensitive for HEV serotypes 1 and 3 in the livers of both immunocompromised and immunocompetent patients[143]. Detecting HEV in liver tissues may be more reliable and may correlate directly with liver inflammation and damage in the immunocompromised. Regardless, a limitation of ORF2 clone 1E6 staining is that it is less sensitive for HEV genotypes 2 and 4. A cost-effective method of detecting HEV infection with high efficacy is still needed. Table 5 summarizes the HEV detection methods and their diagnostic value[144,145].

Antiviral therapy for HEV infection: In addition to ribavirin, other medications used in HEV-infected adults include pegylated interferon-α and add-on effects of sofosbuvir with ribavirin[128,146]. Recent data show that interferon λ1-3 inhibits HEV replication in an *in vitro* culture system and may be effective for treating HEV infections[147]. Another proposed medication is zinc salt. In a human hepatoma cell study, zinc salt dose-dependently inhibited replication of HEV genotypes 1 and 3[148]. In fact, zinc

Table 4 Studies of children infected with hepatitis E virus after liver transplantation

Ref.	Year	Country	Participants	Seroprevalence of HEV infection	Methods		Comments
					HEV IgM/G	HEV RNA	
1[130]	2012	Canada	Gr 1; N: 66 with normal LFT, aged 13.7 yr (1.8-25.5); Gr 2; N: 14 with transaminitis, aged 17.4 yr (5.9-19.8)	Gr 1: 10/66 (15%) with IgG +, none had IgM, HEV RNA +; Gr 2: 12/14 (86%) with IgG+; 9/12 (75%) with IgM+; 1/12 (0.8%) with HEV RNA +	Feldan Bio Inc, Saint-Augustin	Serum nested RT-qPCR	All in Gr 2 showed a trend toward chronic hepatitis and fibrosis; An 8-yr-old girl had chronic HEV infection (genotype 3) for > 10 yr and developed cirrhosis
2[131]	2012	Germany	N: 41 liver-transplanted children, aged 8.8 ± 4.2 yr	2/41 (4.9%) IgG +0/41 stool HEV RNA +	Mikrogen	Stool RT-qPCR	No case with chronic HEV infection
3[132]	2013	Germany	N: 22 liver-transplanted children, aged 6.7 yr (1.4-17.2)	1/22 (0.45%) IgG + by Wantai assay and HEV RNA + in serum	Wantai assay	Serum or stool PCR	10-year-old boy with HEV infection that had persistent transaminitis after 2-mo immunosuppressive reduction. Ribavirin 15 mg/kg/d was started for 6 mo. Normal LFT and undetectable serum and stool HEV RNA at day 42 of treatment.
4[139]	2014	Brazil	One liver-transplanted child: case report	HEV IgG/IgM and HEV RNA in serum and liver tissue at 6-10 yr after liver transplantation	Mikrogen	Liver and serum RT-PCR	A 4-yr-old girl with transaminitis from ACR at 6 yr after LT, had transaminitis off and on and HEV IgG/IgM and HEV RNA was detected 9-10 yr after LT. Chronic HEV infection was successful treatment with ribavirin for 10 mo.
5[133]	2015	France	84 liver-transplanted children, aged 12.3 yr	8/84 (8.3%) HEV IgG+	Wantai assay	Ceeram Tools® kit for HEV-RNA detection	None had HEV IgM/RNA +; No case of chronic infection
6[140]	2020	France	80 liver-transplanted children, aged 3.5 ± 4 yr	6/80 (8%) with HEV IgG+	Wantai assay	Ceeram Tools® kit for HEV-RNA detection	None had HEV IgM/RNA +; No case of chronic infection; 4/6 had undetectable HEV IgG after follow-up (3-42 mo)
7	2021	Thailand	30 liver-transplanted children with transaminitis, aged 1.2-17.6 yr	14/30 (45.2%) with HEV IgG+, 4 (13%) with HEV IgM+ and one case with HEV RNA in stool	Euroimmun kit	Stool PCR	All of them had persistence of HEV IgM from 5 to 44 mo and transaminitis from 4 to 30 mo before HEV testing. The previous treatment included graft rejection, *de novo* autoimmune hepatitis and CMV viremia.

Ref: Reference; Gr: Group; RT-qPCR: Real-time polymerase chain reaction; HEV: Hepatitis E virus; ACR: Acute cellular rejection; MP: MP Biomedicals, formerly Genelabs Diagnostics, Singapore; Wantai assay: Wantai Biologic Pharmacy Enterprise, Beijing, China; LT: Liver transplantation; CMV: Cytomegalovirus.

Table 5 Diagnostic tests for hepatitis E infection[144,145]

Detection	Technique	Specimen
Virus or its components (direct method)	**HEV nucleic acid:** (1) RT-PCR; (2) Realtime RT-PCR; and (3) Loop-mediated isothermal amplification assay. **HEV RNA:** (1) *In situ* hybridization; (2) HEV viral protein (antigen); (3) EIA; and (4) IHC.	Serum, stool, bile, liver tissue
Host immune response (indirect method)	**Specific anti-HEV antibodies (IgM and IgG)** (sensitivity 72%-98% and specificity 78%-96%): (1) Indirect EIA; (2) Immunochromatographic assays; (3) Double-antigen sandwich-based EIAs; (4) μ capture EIAs for IgM anti-HEV; (5) Specific cellular immune response; and (6) ELISpot assays.	Serum, peripheral blood mononuclear cells

HEV: Hepatitis E virus; RT-PCR: Reverse transcription polymerase chain reaction; ELISpot: Enzyme-linked immune absorbent spot; EIA: Electroimmunoassay; IHC: Immunohistochemistry.

can directly decrease HEV replication by suppressing viral translation and processing of nonstructural proteins encoded by ORF1 and by inhibiting IFN-λ3 from binding to its receptor[149,150]. Moreover, zinc has an indirect effect by modulating host immune

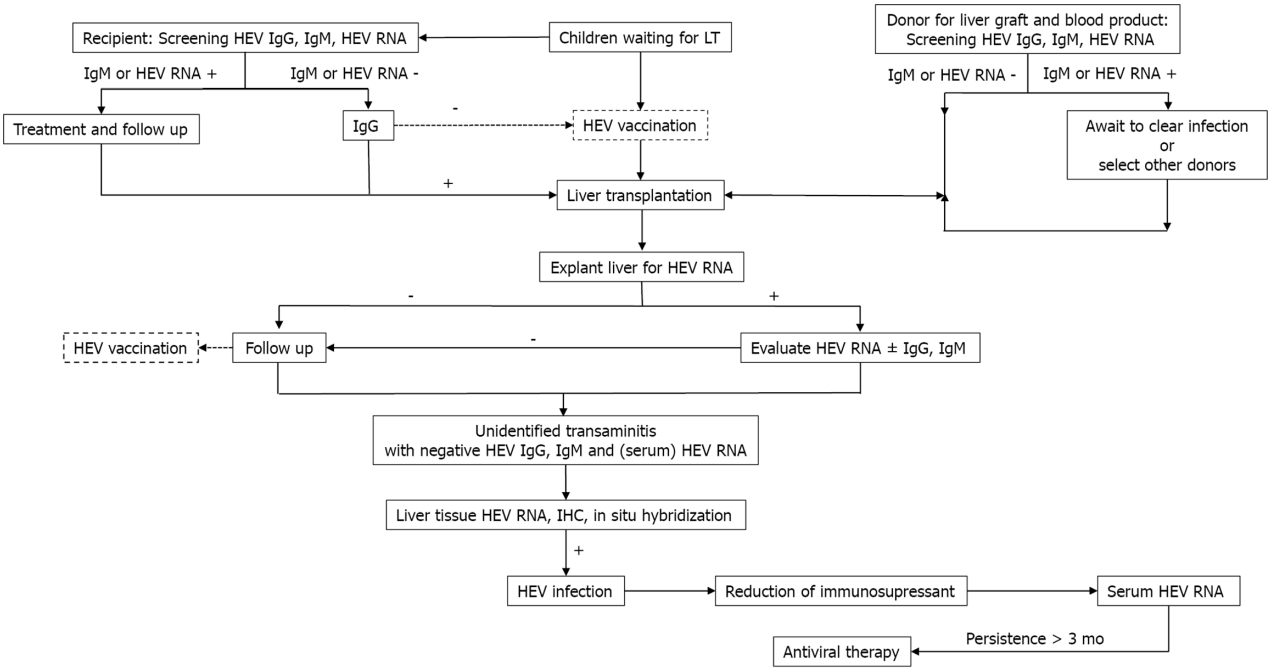

Figure 3 Proposed strategies to eliminate hepatitis E virus infection in children after liver transplants[127,128,130,135,141,142]. LT: Liver transplantation; HEV: Hepatitis E virus; IHC: Immunohistochemistry.

responses and is a cofactor in host cellular processes[150]. Hence, zinc is a promising drug for HEV therapy without serious adverse effects. Clinical and basic research are needed regarding the therapeutic benefits of zinc in HEV infections.

Prevention with an HEV vaccine: Since 2001, several vaccines based on virus-like particles have been developed[151], and there have been clinical trials on three vaccine candidates[136,137,152]. One is licensed in China, with 100% efficacy over 12 mo after 3 injections[137]. Moreover, the efficacy remained high at 86.8% after a 4.5-year follow-up[138]. However, these three vaccines mainly protect against genotypes 1 and 4 but cannot protect against genotype 3, which is the main genotype causing chronic HEV infections in patients after LT[153]. In 2019, an HEV vaccine was initiated and is progressing in clinical trials in the United States[153]. In general, an HEV vaccine will be a powerful weapon in public health for protecting against HEV infections (Figure 3).

CONCLUSION

To eliminate viral hepatitis in paediatric liver-transplant recipients, multiple strategies must be integrated into clinical practice. Similar to the prevention of HAV infections, immunization is the mainstay of prevention against HBV infection in children with liver transplants. Regular monitoring of humoral immunity for HBV and HAV and revaccination programmes in cases with immunity loss are necessary. Antiviral therapy plays a major role in HBV and HCV infections. For HEV infection, molecular techniques for early detection in children with liver transplant with unidentified causes of hepatitis should be developed to guide proper management of HEV infection.

ACKNOWLEDGEMENTS

We would like to express our sincere respect and gratitude to Professor Poovorawan Y, our mentor and friend, for devoting nearly all of his life to Thai research development. He is our role model and truly inspires us to conduct more bench and bedside integrated research in the future.

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World Journal of Gastroenterology

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World J Gastroenterol 2022 January 21; 28(3): 310-331

DOI: 10.3748/wjg.v28.i3.310

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

REVIEW

Non-alcoholic fatty liver disease and hepatocellular carcinoma: Clinical challenges of an intriguing link

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Author contributions: Chrysavgis L performed the investigation, conceptualization, writing-original draft, visualization; Giannakodimos I and Diamantopoulou P contributed to the writing-original draft, and validation; Cholongitas E performed the resources, writing-review and editing, supervision, project administration; all authors have read and agreed to the final version of the manuscript.

Conflict-of-interest statement: The authors have nothing to disclose related to the manuscript.

Country/Territory of origin: Greece

Specialty type: Gastroenterology and hepatology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): A

Grade B (Very good): B, B

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Abstract

Non-alcoholic fatty liver disease (NAFLD) has emerged as the most common liver disorder worldwide mainly attributed to the epidemic spread of obesity and type 2 diabetes mellitus. Although it is considered a benign disease, NAFLD can progress to non-alcoholic steatohepatitis, liver cirrhosis and hepatocellular carcinoma (HCC). Most data regarding the epidemiology of NAFLD-related HCC are derived from cohort and population studies and show that its incidence is increasing as well as it is likely to emerge as the leading indication for liver transplantation, especially in the Western World. Although cirrhosis constitutes the main risk factor for HCC development, in patients with NAFLD, HCC can arise in the absence of cirrhosis, indicating specific carcinogenic molecular pathways. Since NAFLD as an underlying liver disease for HCC is often underdiagnosed due to lack of sufficient surveillance in this population, NAFLD-HCC patients are at advanced HCC stage at the time of diagnosis making the management of those patients clinically challenging and affecting their prognostic outcomes. In this current review, we summarize the latest literature on the epidemiology, other than liver cirrhosis-pathogenesis, risk factors and prognosis of NAFLD-HCC patients. Finally, we emphasize the prevention of the development of NAFLD-associated HCC and we provide some insight into the open questions and issues regarding the appropriate surveillance policies for those patients.

Key Words: Non-alcoholic fatty liver disease; Hepatocellular carcinoma; Epidemiology; Risk factors; Surveillance; Risk stratification

Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

Received: September 9, 2021
Peer-review started: September 9, 2021
First decision: October 16, 2021
Revised: October 19, 2021
Accepted: January 6, 2022
Article in press: January 6, 2022
Published online: January 21, 2022

P-Reviewer: Lu XL, Tunsophon S, Zhou J
S-Editor: Fan JR
L-Editor: A
P-Editor: Fan JR

Core Tip: Non-alcoholic fatty liver disease (NAFLD) is projected to emerge as the leading cause of hepatocellular carcinoma (HCC) worldwide. Demographic factors, genetic predisposition and behavioral parameters have been identified as independent risk factors for NAFLD-related HCC, which can arise even in the absence of cirrhosis. Currently, the most challenging issue for the scientific community worldwide is the identification of the pre-cirrhotic NAFLD patients who have increased risk for HCC. Noteworthy, the central concept for the surveillance policies in the near future should be the identification, *via* an individual, risk-assessment based precision screening of high-risk NAFLD patients, cirrhotic or not.

Citation: Chrysavgis L, Giannakodimos I, Diamantopoulou P, Cholongitas E. Non-alcoholic fatty liver disease and hepatocellular carcinoma: Clinical challenges of an intriguing link. *World J Gastroenterol* 2022; 28(3): 310-331
URL: https://www.wjgnet.com/1007-9327/full/v28/i3/310.htm
DOI: https://dx.doi.org/10.3748/wjg.v28.i3.310

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is defined as the presence of triglycerides ≥ 5% into the hepatic tissue (*i.e.* steatosis), in the absence of excessive alcohol consumption and other competing liver disorders such as chronic viral hepatitis or administration of steatogenic drugs[1]. The disease may progress to non-alcoholic steatohepatitis (NASH) which is characterized by steatosis and liver inflammation, with or without fibrosis. NAFLD is considered as the global epidemic of the 21st century in the field of liver diseases and is strongly associated with the increased prevalence of obesity[1,2]. In 2016, the World Health Organization estimated the number of overweight or obese adults to be more than 1.9 billion worldwide. NAFLD is also correlated with other metabolic comorbidities, besides obesity, namely type 2 diabetes mellitus (T2DM), hyperlipidemia, arterial hypertension and it is considered the hepatic manifestation of the metabolic syndrome (MetS)[3]. Concerning epidemiology of NAFLD, a recent large meta-analysis, including 45 studies reported an estimated global prevalence of NAFLD as high as 25.24% with highest prevalence in the South America (30.45%) and Middle East (31.8%) and lowest in Africa (13.5%)[4]. Of note, during the past decade, a consistent rise of NAFLD prevalence was observed, increasing from 15% in 2005 to 25% in 2010[4].

Concerning the advanced form of the disease, the pooled NASH prevalence among NAFLD patients with an indication for liver biopsy was 63.45% for Asian region, 69.25% for Europe and 60.64% for North America[4]. Oppositely, NASH prevalence among NAFLD patients without an indication for biopsy was 6.67% for Asia and 29.85% for North America, while no corresponding data were available for European territory[4]. Moreover, approximately 41% of NASH patients experienced fibrosis progression with an average annual progression rate of 0.09%[4]. A smaller, but significant proportion of NAFLD and mainly NASH patients will ultimately develop cirrhosis or even hepatocellular carcinoma (HCC), thus facing life-threatening liver-associated complications[5]. To our point of view, since prevalence of NAFLD is increasing rapidly globally, HCC in patients with NAFLD will become a major public health issue and will emerge as a leading cause for liver transplantation (LT) in the near future. The vast underestimation of the true burden of NAFLD, especially in the developing countries, leads to reduced and delayed access of patients to specialized medical centers for appropriate surveillance and treatment of HCC and its complications, since early diagnosis constitutes a fundamental factor for effective therapy.

In this current review, we discuss the latest data concerning epidemiology, risk factors and prognosis of NAFLD-related HCC as well as we emphasize the prevention, and the appropriate surveillance polices which shall be conducted to improve patients' attentiveness and care.

LITERATURE SEARCH

We reviewed the current literature from the inception of this current review until March 2021. For our scope, we used "PubMed" database and we included only studies written in English. We used the following search terms: "Non-alcoholic fatty liver disease-related hepatocellular carcinoma", "NAFLD-related HCC", "NAFLD-HCC", "Non-alcoholic steatohepatitis-related hepatocellular carcinoma", "NASH-related HCC", "NASH-HCC" and we retrieved the results of our search for the epidemiology, pathogenesis, risk factors, prognosis/outcomes, surveillance, and prevention of NAFLD/NASH-related HCC. Also, the references of the research articles were scrutinized for relevant studies.

EPIDEMIOLOGY OF NAFLD-RELATED HCC

HCC, as an entity, is the fifth most frequently diagnosed cancer and the second leading etiology of cancer-related mortality worldwide[6]. It is the predominant histological type of primary liver cancer accounting for 70%-85% of all liver cancer cases[6] and is estimated to be the fastest growing cause of cancer-related mortality among United States male population[7]. Although most HCC cases occur in the setting of chronic viral hepatitis or alcoholic (ALD) cirrhosis, a significant proportion of patients with NAFLD/NASH may develop HCC. According to the aforementioned recent meta-analysis, the annual incident rate of HCC in NASH patients was 5.29 *per* 1000 person-years (PY), whereas for NAFLD patients that percentage dropped to 0.44 *per* 1000 PY[4]. Noteworthy, another meta-analysis which included only studies of Asian populations reported that the incidence rate of HCC was 1.8 cases *per* 1000 PY on NAFLD patients, while the corresponding data for NASH patients were unavailable due to the design of that meta-analysis[8]. The prevalence of NAFLD-related HCC is rising worldwide, and data derived from studies conducted in the past decade estimated that 4%-22% of all HCC were attributed to NAFLD in Western countries with the corresponding percentage to be 1%-2% in Asian region, where viral hepatitis remains endemic[9]. However, these studies underestimate the true NAFLD-related HCC prevalence, as they ignore the impact of "cryptogenic" cirrhosis. It is widely suspected that half of the cryptogenic cirrhosis-related HCC, which accounts for 15% to 30% of all HCC cases, arise from NAFLD[10].

Regarding the prevalence of HCC among NASH patients (cirrhotic or not), as compared to other liver diseases, a recent meta-analysis demonstrated that it was 22.5% for all NASH and 13.6% among all other non-NASH patients[11]. More recently, a large health-care database study in the United States identified NAFLD or NASH as the most predominant underlying risk factor for HCC, being present in 59% of cases [12]. In addition, NAFLD accounted for 34.8% of HCC events in England, while a year-by-year increase of HCC attributed to NAFLD between 2000 and 2010 was identified [12,13]. In a recent analysis of the Scientific Registry of Transplant Recipients including data from 158347 candidates for LT in the United States between 2002 and 2016, the proportion of NAFLD/NASH in HCC was increased 7.7-fold (from 2.1% to 16.2%), while the corresponding proportion for other-etiologies HCC remained relatively stable[14]. Of note, during this period the prevalence of HCC in LT candidates with NASH increased 11.8-fold[14]. Consistent with that, during a 20-year period, among a French cohort of histologically confirmed HCC patients who underwent surgical resection, the prevalence of NAFLD-related HCC increased from 2.6% in the period of 1995-1999 to 19.5% in 2010-2014, while the corresponding hepatitis C virus (HCV)-related fraction was decreased from 43.5% to 19.5%[15]. Along this line, Hester *et al*[16] in their recent cross-sectional study including 13648 HCC patients, identified NAFLD as the predominant cause of HCC in both inpatient and outpatient population, accounting for 32.07% and 20.22% of all cases respectively, followed by HCV infection [16].

Of note, a major cause for concern is the incidence of HCC among T2DM patients considering both the high prevalence of T2DM globally and the fact that > 70% of T2DM patients have NAFLD[17]. A large observational study revealed that HCC was the most incident malignancy among 457473 T2DM patients (Hazard Ratio: 3.31), while a large population-control study further confirmed that T2DM was independently associated with 2-3-fold increase of HCC risk regardless of other well-established risk factors for HCC[18]. Interestingly, Dyson *et al*[13] showed that the prevalence of T2DM or obesity among HCC patients was growing during a decade of follow-up (2000-2010) while, intriguingly, in one third of all HCC patients referred to

this tertiary care centre, metabolic dysregulation was the only identified risk factor for HCC[13]. Of importance, numerous meta-analyses have also demonstrated similar findings[19-21]. We should emphasize that throughout the above-mentioned meta-analyses, the association between T2DM and HCC was robust across different population groups, geographic areas, and a plethora of control groups while it remained significant even after adjusting for demographic and laboratory parameters.

Of cardinal importance, a distinctive feature of NAFLD/NASH, compared to other liver diseases such as HCV or ALD, is the development of HCC even in the absence of cirrhosis[22]. In a retrospective study of 1500 patients with HCC, the incidence of HCC development without cirrhosis was higher in NAFLD patients, since 34.6% of NAFLD-HCC patients were non-cirrhotic, while only 8.9% in HCV, 7.7% in hepatitis B virus (HBV) and 11.1% in ALD groups had no evidence of cirrhosis[23]. Consistently, two large independent Western studies showed that 54% and 46.2% of NAFLD and NASH-related HCCs respectively, arose in a non-cirrhotic background[12,24], while the corresponding proportion was similar (49%) in a cross-sectional multicenter Japanese study[25]. Additionally, a recent meta-analysis confirmed those findings since in non-cirrhotic NASH subjects the pooled prevalence of HCC was 38% compared to 14.2% in non-cirrhotic non-NASH[11] suggesting that the former had significantly increased odds for developing HCC[11]. Thus, NAFLD seems to be the second cause, together with HBV, where HCC can develop in non-cirrhotic liver. Studies of the last decade concerning the epidemiology of NAFLD-related HCC are summarized in Table 1[13,15,16,23,26-45], while studies including cohorts of NAFLD patients who prospectively developed HCC are summarized in Table 2[24,25,46-52].

PATHOGENETIC PATHWAYS

Like other malignancies, NAFLD-related HCC development is a chronic process with gradual transition from the state of NAFLD to the state of cirrhosis and HCC onset. In the setting of liver cirrhosis, the pathophysiological mechanisms of HCC arising have been well-studied. Repeated cycles of hepatocyte death and subsequent liver regeneration and tissue restoring along with cellular proliferation and constant cell growth lead to tumor development[53]. Besides hepatic cirrhosis, in this current review we shed light to several other etiologies and mechanisms that have been specifically implicated in the development of NAFLD-related HCC, since a lot of non-cirrhotic related HCC are associated with NAFLD[36]. The potential pathogenetic molecular pathways implicated in the NAFLD-related HCC development are illustrated in Figure 1 (Created with BioRender https://biorender.com/). Herein, we highlighted the major and well-established pathogenetic mechanisms whereas the remaining and relatively recently proposed ones are in detail described in the Supplementary material.

NAFLD is closely associated with insulin resistance and subsequently increased levels of insulin and insulin-like growth factor-1 (IGF-1)[54]. Binding of these two molecules to insulin receptor and insulin-like growth factor-1 receptor (IGF1R) respectively, results in activation of PI3K/AKT and Mitogen-activated protein kinase (MAPK) molecular pathways[55]. Regarding the first, exerts its action by signaling on cyclin D1, Mdm2/p53 and mTOR and leads to inhibition of apoptosis, induction of cell proliferation and excessive cell growth respectively, while the activation of MAPK mediates the transcription of proto-oncogenes c-fos and c-jun, further affecting cell growth[56]. Moreover, MAPK pathway facilitates the activation of Wnt/β-catenin signaling cascade, which leads to liver fibrosis and promotion of hepatocarcinogenesis [57]. Moreover, insulin resistance and energy imbalance drive to excessive liver lipid accumulation, metabolic reprogramming and production of free fatty acids (FFAs) [58]. Increased mitochondrial oxidation of these FFAs induces the formation of reactive oxygen species (ROS) leading to insufficient mitochondrial respiratory chain activity as well as triggering apoptotic death pathways, such as receptor interacting protein 1 (RIP1) and RIP3- activated Jun-(N)-terminal kinase (JNK),which in turn facilitate liver inflammation and fibrosis[59,60]. In addition, the increased FFA oxidation is associated with augmented levels of endoplasmic reticulum (ER) and oxidative stress in hepatocytes. The latter promotes increased calcium release from ER that leads to mitochondrial permeabilization, disrupted ER function, liver cell injury and tumorigenesis in NASH[61]. Moreover, the crosstalk between oxidative or ER stress and ROS overproduction aggravates the progression of liver disease into NASH and HCC as the aforementioned mitochondrial dysfunction leads to further overproduction of ROS, which facilitate the activation of proapoptotic paths, mediating by

Table 1 Epidemiology of non-alcoholic fatty liver disease-related hepatocellular carcinoma based on studies published in the last decade (2011-2020)

Ref.	Year/Country	Study design	HCC patients, *n*	HCC caused by NAFLD, *n* (%)	Non-cirrhotics among HCC-related NAFLD, *n* (%)	Fibrosis stage of NAFLD-HCC patients, *n* (%)	Tumor size
Yang *et al* [26]	2011/United States	Retrospective	460	61 (13.27)	NA	NA	NA
Schütte *et al* [27]	2014/Germany	Retrospective	664	43 (6.5)	6 (13.95)	NA	NA
Chun *et al* [28]	2014/United States	Retrospective	27	13 (48.1)	NA	NA	NA
Edenvik *et al*[29]	2015/Sweden	Retrospective	616	69 (11.2)	15 (21.7)	NA	NA
Younossi *et al*[30]	2015/United States	Retrospective	4979	701 (14.1)	NA	NA	NA
Weinmann *et al*[31]	2015/Germany	Retrospective	1119	45 (4)	10 (22.2)	NA	Trend towards ↑ tumor size in NASH-HCC (6 cm) *vs* non-NASH-HCC (4.8 cm) (P = 0.18)
Mittal *et al* [23]	2016/United States	Retrospective	1500	107 (8)	37 (34.6)	NA	NA
Wong *et al* [32]	2017/United States	Retrospective	17.664	5898 (33.4)	3326 (56.4%)	NA	↑ proportion of tumors > 5 cm in NAFLD-HCC *vs* non-NAFLD-HCC (P < 0.001)
Huang *et al* [33]	2017/Australia	Prospective	270	38 (14)	9 (23.7)	NA	NA
Koh *et al*[34]	2019/Singapore	Prospective	996	152 (15.3)	100 (65.8)	F0 = 78 (51.7); F1 = 10 (6.6); F2 = 45 (29.8); F3 = 9 (6); F4 = 9 (6)	↓ tumor size in NAFLD-HCC (0.7 cm) *vs* non-NAFLD-HCC (4 cm) (P < 0.001)
Hassan and Gane[35]	2019/New Zealand	Retrospective	1985	159 (5.1) (Undefined cirrhosis stage in 57)	25 (24.5) (based on well-defined stage patients)	F0 = 2 (8); F1 = 3 (14); F2 = 1 (3); F3/4 = 19 (75.5)	NA
Gawrieh *et al*[36]	2019/United States	Retrospective	5144	767 (14.9)	159 (26.3)	NA	NA
Hester *et al* [16]	2020/United States	Retrospective	12471	3019	1565	NA	NA
Hong *et al* [37]	2018/Australia	Prospective	272	39 (14.3)	NA	NA	NA
Jamwal *et al* [38]	2020/India	Prospective	56	20 (35.7)	20 (100)	NA	NA
Pais *et al*[15]	2017/France	Retrospective	323	39 (12.1)	30 (76.9)	F0 = 16 (40); F1 = 9 (23); F2 = 0 (0); F3 = 5 (14); F4 = 9 (23)	↑ tumor size in NAFLD-HCC (8.7 cm) *vs* non-NAFLD-HCC (6.2 cm) (P = 0.002)
Dyson *et al* [13]	2013/United Kingdom	Prospective	632	136 (21.5)	31 (22.8)	NA	NA
Phipps *et al* [39]	2020/United States	Retrospective	5327	790 (14.8)	NA	NA	NA
Bengtsson *et al*[40]	2019/Sweden	Retrospective	1562	225 (14.4)	83 (36.9)	F0 = 1; F1 = 13; F2 = 16; F3 = 5 (Undefined fibrosis stage in 48 patients)	NSD in tumor size between NAFLD-HCC *vs* non-NAFLD-HCC; ↑ tumor size in non-cirrhotic *vs* cirrhotic NAFLD-HCC (P = 0.001)
Tokushige *et al*[41]	2013/Japan	Retrospective	14.530	292 (2)	111 (38)	NA	NA
Reddy *et al* [42]	2012/United States	Retrospective	303	52 (NASH) (17.2)	14 (26.9)	NA	NSD in tumor size between NAFLD-HCC (3.2 cm) *vs*

							non-NAFLD-HCC (3 cm)
Phan *et al* [43]	2019/United States	Retrospective	545	28 (5.1)	3 (10.7)	NA	NA
Van Meer *et al*[44]	2016/Netherlands	Retrospective	1221	181 (14.8)	67 (28)	NA	↑ tumor size in NAFLD-HCC (6 cm) *vs* HCV-HCC (3 cm) ($P < 0.001$)
Yang *et al* [45]	2017/United States	Retrospective	93	10 (11)	3 (27.3)	NA	NA

NAFLD: Non-alcoholic fatty liver disease; HCC: Hepatocellular carcinoma; NASH: Non-alcoholic steatohepatitis; HCV: Hepatitis C virus; NSD: No significant difference; NA: Not applicable.

caspases 9 and 3[62,63].

Furthermore, the products from lipid peroxidation and the elevated levels of ROS provoke the release of several pro-inflammatory and inflammatory substances such as interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) as well as affects adipokines' secretion, namely leptin and adiponectin[64]. Increased expression of IL-6 activates the oncogenic pathway of signal transducer and activator of transcription 3 (STAT-3) which mediates cell proliferation, inhibits apoptosis and contributes to HCC development, while augmented TNF-α levels mediate the activation of pro-oncogenic paths namely nuclear factor kB (NF-kB) *via* JNK and phosphorylation of inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta (IKKβ)[65]. Consistently, leptin, as a profibrotic and proangiogenic factor exerts its action by both stimulating an intracellular signaling cascade of the inflammatory molecules namely TNF-α and IL-6 and by activating the previously mentioned JAK2/STAT-3, MAPK and PI3K pathways upon its binding to its receptor in HCC cells[66]. Noteworthy, although adiponectin is a strong anti-inflammatory mediator which modulates apoptosis under normotropic conditions, the exacerbated insulin resistance suppresses its action while ROS-induced overproduction of leptin, as an antagonistic hormone on the field of hepatic fibrogenesis with adiponectin, further inhibits its production and thus intensifies HCC development[67,68].

As the activation of the immune system considered as a prerequisite for NAFLD progression to NASH, the involvement of immunological pathways in the NAFLD-related HCC is of much interest. Ma *et al*[69] reported that selective intrahepatic depletion of CD4+ T cells robustly induced tumor development in a methionine-choline-deficient liver specific MYC transgenic mouse showing that CD4+ T cells mediate tumor regression[69]. Moreover, stimulation of hepatocellular lymphotoxin-β receptor (LTBR) and NF-kB signaling led to HCC onset in a MCD high fat diet mouse model whereas that same dietary pattern induced activation of natural killer T (NKT) cells and intrahepatic CD8+ T cells, which in turn facilitated NASH to HCC transition [70]. Additionally, liver damage and subsequent inflammatory response leads to activation of Kupffer cells (KCs), which are the resident macrophages of the liver and their involvement into NAFLD progression is well established in both animal models and human hepatic dysregulation[71]. Yet, these cells are also implicated in the hepatocarcinogenesis since they express the pro-inflammatory myeloid cell surface receptor TREM1 which facilitates HCC development in a diethyl nitrosamine-induced HCC mouse model[72]. KCs also express Toll-like receptor 4 and binding of Lipopolysaccharides drives the activation of the above-mentioned tumorigenic pathways of NF-kB, JNK and MAPK[73]. Additionally, upon acute liver cells injury, the signaling pathway Hedgehog was triggered and reinforced the recruitment of hepatic progenitor cells at the sites of injury in order to replace the damaged hepatocytes[74]. Dysregulated signaling of this pathway leads to insufficient cell repair within the hepatic parenchyma and results in malignancy and HCC progression[75].

RISK FACTORS

Although cirrhosis constitutes the major risk factor for the development of HCC in various liver diseases, including NAFLD, HCC can also occur in non-cirrhotic NAFLD individuals[76]. Demographic, behavioral, or genetic factors contribute along with cirrhosis or even more in absence of cirrhosis to HCC. Older age, male sex and Hispanic ethnicity are strongly associated with higher risk of HCC development[5]. In a cohort study of 296707 NAFLD patients, age over 65 years comprised an

Table 2 Characteristics of non-alcoholic fatty liver disease-associated hepatocellular carcinoma based on studies including cohorts of non-alcoholic fatty liver disease-associated hepatocellular carcinoma patients

Ref.	Year/Country	Total NAFLD-HCC patients	Prevalence of NAFLD-HCC without cirrhosis, *n* (%)	Fibrosis stage of non-cirrhotic NAFLD-HCC patients, *n* (%)	Tumor characteristics in cirrhotic *vs* non-cirrhotic NAFLD-HCC patients (differentiation)
Piscaglia *et al* [24]	2015/Italy	145 patients	67 (46)	F0 = 3 (18.75); F1-F2 = 2 (12.5); F3 = 11 (68.75) (Undefined fibrosis stage in 51 patients)	NSD in tumor size
Leung *et al*[46]	2015/Australia	54 patients	8 (15)	F0 = 2 (33.3); F1-F2 = 4 (66.7) (Undefined fibrosis stage in 2 patients)	↑ tumor diameter in non-cirrhotic (4.7 cm) *vs* cirrhotic (3.2 cm) (P = 0.041). NSD in median number of tumors in non-cirrhotic (2) *vs* cirrhotic (1). NSD in HCC differentiation
Kodama *et al* [47]	2019/Japan	104 patients	58 (55.8)	F0 = 6 (5.8); F1 = 11 (10.6); F2 = 18 (17.3); F3 = 23 (22.1)	NSD in HCC differentiation
Mohamad *et al*[48]	2015/United States of America	83 patients	36 (43.4)	F0 = 18 (55.9); F1 = 6 (17.6); F2 = 3 (8.8); F3 = 6 (17.6)	↑ incidence of single nodules in non-cirrhotic (80.6%) *vs* cirrhotic (52.2%) (P < 0.05). ↑ proportion of large nodule size (> 5 cm) in non-cirrhotic (77.8%) *vs* cirrhotic (10.6%) (P < 0.05). NSD in HCC differentiation
Tobari *et al* [49]	2020/Japan	119 patients	48 (40.3)	F0-F1 = 12 (32.4); F2 = 17 (46); F3 = 8 (21.6) (Undefined fibrosis stage in 11 patients)	↑ tumor size in non-cirrhotic (46 mm) *vs* cirrhotic (28 mm) (P < 0.01). NSD in HCC differentiation. NSD in median number of tumors
Yasui *et al*[25]	2011/Japan	87 patients	43 (49.4)	F1 = 10 (23.2); F2 = 15 (34.9); F3 = 18 (41.9)	NA
Thompson[50]	2018/United States	48 patients	26 (54)	F0 = 10 (38.5); F1 = 8 (30.8); F2 = 5 (19.2); F3 = 1 (3.8)	↓ tumor size in non-cirrhotic (3.3 cm) *vs* cirrhotic (5.7 cm) (P < 0.01). NSD in HCC differentiation
Cotrim *et al* [51]	2016/Brazil	110 patients	20 (48.5)	F0 = 2 (12.5); F1-3 = 14 (87.5)	NA
Iannaccone *et al*[52]	2007/France	22 patients	16 (72.3)	F0 = 7 (31.8); F1-3 = 9 (40.9)	NA

NAFLD: Non-alcoholic fatty liver disease; HCC: Hepatocellular carcinoma; NSD: No significant difference; NA: Not applicable.

independent risk factor for HCC occurrence[77]. In the same study, the incidence of HCC was higher in males compared to females (0.22 *vs* 0.04 *per* 1000 PY respectively), in Latino *vs* White and African-American patients (0.29 *vs* 0.21 and 0.12 *per* 1000 PY, respectively) and in cirrhotic compared to non-cirrhotic patients (10.2 *vs* 0.02 *per* 1000PY, respectively)[77].

Furthermore, distinctive MetS-related features, namely obesity and T2DM have been identified as risk factors for HCC[78]. In a meta-analysis, overweight and obese patients had 17% and 89% increased relative risk for HCC respectively compared to normal-weight individuals[79]. In another study, obesity was recognized as an independent predictor for HCC development only in patients with cryptogenic [odds ratio (OR): 11.1; 95% confidence interval (CI): 1.5-87.4] and ALD-related cirrhosis (OR: 3.2; 95%CI: 1.5-6.6)[80]. Concerning the burden of T2DM, in a retrospective study including 6508 NAFLD patients, T2DM comprised an independent risk factor for the

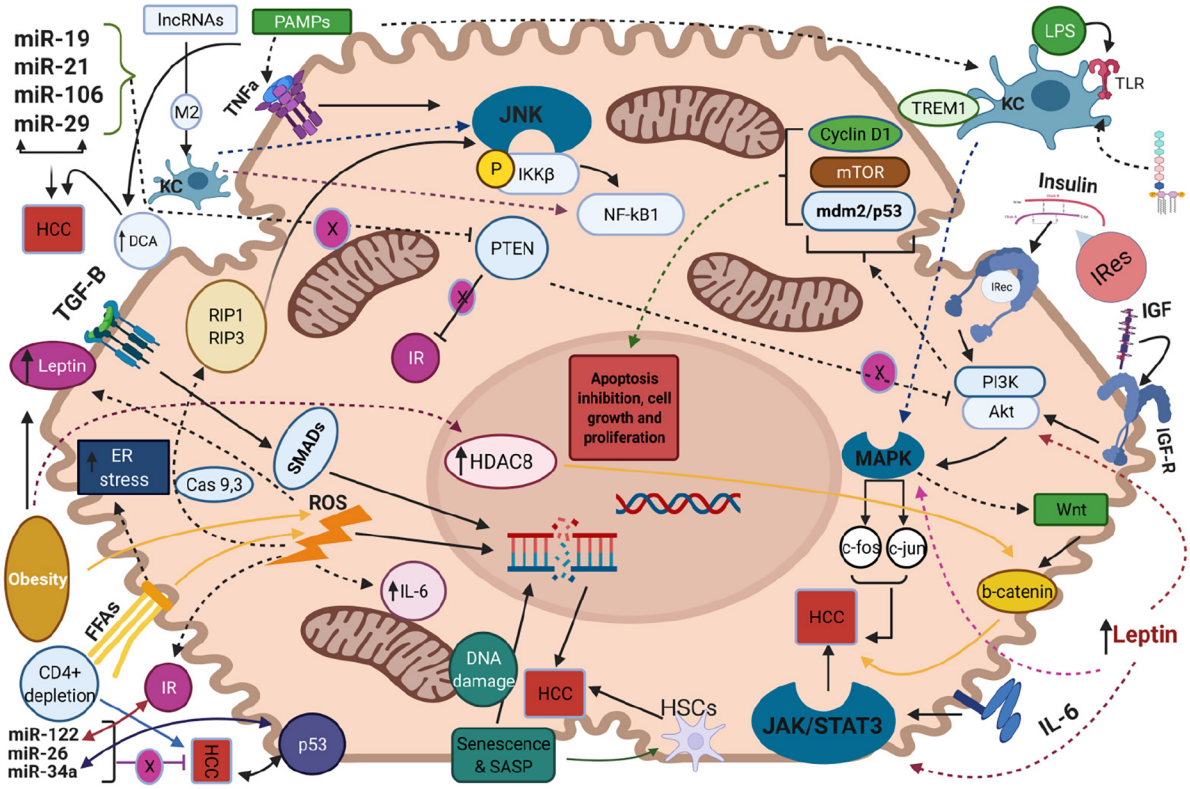

Figure 1 The molecular pathways that regulate non-alcoholic fatty liver disease-related hepatocellular carcinoma along with their interactions are represented. Activation of downstream signaling pathways is indicated by full or dot lines whereas inhibition of them is indicated by blunted lines with a circular-shaped "X". Bidirectional arrows highlight the interplay between distinct molecular pathways. Molecules acting as mediators of signaling paths are indicated over a full, dot or blunted line in a box along with their names. MicroRNAs that promote hepatocellular carcinoma development are collectively represented in the upper left of the figure while tumor suppressor microRNAs are represented in the lower left of the schema. Cas: Caspase; DCA: Deoxycholic acid; ER: Endoplasmic reticulum; FFAs: Free fatty acids; HCC: Hepatocellular carcinoma; HDAC8: Histone deacetylase 8; HSCs: Hepatic stellate cells; IGF: Insulin growth factor; IL-6: Interleukin-6; IRec: Insulin receptor; IRes: Insulin resistance; JNK: Jun-(N)-terminal kinase; KC: Kuppfer cell; lncRNAs: Long non-coding RNAs; LPS: Lipopolysaccharides; MAPK: Mitogen-activated protein kinase; PAMPs: Pathogen-associated molecular patterns; SASP: Senescence associated secretory phenotype; STAT3: Signal transducer and activator of transcription 3; TGF-B: Transforming growth factor-B; TLR: Toll-like receptor.

HCC (Hazard ratio: 3.21; 95%CI: 1.09-9.50)[81], while in a Mayo clinic study with 354 NASH-cirrhotic patients, T2DM along with older age and decreased serum albumin levels were identified as independent risk factors for HCC[82]. Consistently, in a case-control study of 185 HCC cases and 404 controls, T2DM (OR: 4.33, 95%CI: 1.89–9.86) and obesity (OR: 1.97, 95%CI: 1.03–3.79) were associated with increased HCC risk, while the combination of obesity and T2DM further exacerbated the hazard of HCC (OR: 4.75, 95%CI: 1.75-12.89)[83]. However, it should be noted that evaluating the exact independent pathogenetic burden of T2DM or obesity in the development of HCC can be really challenging, due to the strong association of these two entities[84].

Moreover, lifestyle modifiable factors, such as smoking and alcohol consumption seem to be implicated in NAFLD-related HCC[84]. In a meta-analysis of 81 studies, the risk for HCC development was higher for both current and former smokers[85], but no specific data were given regarding the relationship between smoking and the incidence of HCC in NAFLD patients[84]. Alcohol consumption is independently related to elevated risk for HCC in NAFLD patients[86], despite that some studies imply that the higher HCC risk is limited only on heavy alcohol use (*e.g.* > 50 g/d[87, 88]), during which NAFLD is excluded by definition. Intriguingly, Sookoian *et al*[89] in their meta-analysis, showed that moderate alcohol consumption (< 30 g/d) is associated with decreased incidence of NAFLD occurrence[89], whereas a prospective study demonstrated a potential synergism between obesity and alcohol intake in increasing the risk of HCC[90]. However, the evidence from those studies could be hindered by potential biases, such as the observational study design which does not permit to ascertain causality, the implication of obesity as potential confounder and the overall insufficient alcohol intake assessment. Overall, the impact of moderate alcohol consumption in NAFLD-related HCC is still difficult to be clarified.

Noteworthy, genetic predisposition further aggravates the risk for NAFLD-related HCC. The possession of Patatin-like phospholipase domain-containing protein 3 (PNPLA3) rs738409C>G and Membrane Bound O-Acyltransferase Domain Containing 7rs641738 polymorphism were independently correlated with increased risk of HCC [91], with the latter being a burdened factor particularly in non-cirrhotic NAFLD patients[92]. On the contrary, a loss of function of variant rs72613567 in 17-beta-hydroxysteroid dehydrogenase 13 has been recently identified to protect against HCC development[93].

PROGNOSIS OF NAFLD-RELATED HCC

The prognostic outcomes of NAFLD-HCC patients as compared to their non-NAFLD-HCC counterparts have been evaluated by population-based and cohort studies with controversial findings (Table 3)[15,24,30,34,40,42,94-103].

Based on Surveillance, Epidemiology and End Results Medicare database, Younossi *et al*[30] performed a large retrospective cohort study including 4979 HCC patients and showed that the one-year mortality risk was significantly higher in NAFLD-HCC compared to HCV/HBV-HCC patients[30]. On average, the former had almost 5 mo shorter survival time compared to the latter[30]. Moreover, in the multivariable model adjusted for clinical and tumour-related parameters, NAFLD was an independent factor associated with increased one-year mortality risk[30]. More recently, Golabi *et al* [94] based on the afore-mentioned database along with outpatients files, showed that NAFLD-HCC patients displayed markedly higher risk of 2-year mortality in comparison with patients with HCV-HCC[94]. Consistent to the abovementioned study, the magnitude of this association remained significant in the multivariable analysis[94]. The afore-mentioned findings were mainly attributed to the more advanced tumour stage at the time of diagnosis due to less intense surveillance of NAFLD patients[94]. In addition, due to the presence of increased visceral obesity, the ultrasonography, which is the current HCC screening tool, may fail to distinguish small tumours in NAFLD patients[94]. Furthermore, the increased prevalence of cardio-metabolic comorbidities along with higher age at the time of HCC diagnosis of NAFLD population may also contribute to the lower possibility of receiving LT, compared to HCC patients with viral hepatitis, and to their decreased survival time [94]. In a prospective study in Italy, among 756 HCC patients the crude 1-year and 3-year overall survival (OS) was remarkably lower in the NAFLD-HCC patients compared to HCV-HCC cohort[24]. In order to account for the potential biases of less intense surveillance and later detection of the NAFLD-related malignancy, they adjusted the whole HCC cohort of patients for the lead time who were under surveillance[24]. Intriguingly, both the mean 1-year and 3-year survival time of NAFLD-HCC patients remained significantly lower than the corresponding of HCV-HCC patients[24]. Along this line, more recently, Hester *et al*[96] in their multivariable analysis demonstrated that the NASH-HCC was associated with worse OS compared only to the ALD-HCC[96]. Additionally, since the magnitude of NAFLD in cryptogenic cirrhosis is major, it is of interest that Giannini *et al*[97] using data form the ITALICA database, showed that patients with cryptogenic cirrhosis-related HCC displayed significantly shorter survival time compared to HCV-HCC patients during a median follow-up of 21 mo[97].

Of importance, we should note that a selection bias within clinical studies could be implicated concerning the worse prognostic outcomes of NAFLD-HCC patients in comparison to other-aetiologies-HCC. Patients who were eligible for radical surgical treatments or LT, were subsequently enrolled in the cohort studies whereas patients with major comorbidities, cardiovascular diseases or advanced age, aspects more common among NAFLD patients, were excluded.

On the contrary, numerous studies have emphasized the better prognosis of NAFLD-HCC patients, compared to other aetiologies of HCC. In a prospective study from Singapore, 844 non-NAFLD-HCC and 152 NAFLD-HCC patients who underwent total liver resection were enrolled and the latter displayed significantly increased 5-year OS as compared to the former, whereas NAFLD was independently associated with lower hazard for mortality in a multivariable model adjusted for clinical and epidemiological parameters[34]. Consistently, after a median follow-up of 50 mo, Reddy *et al*[42] evaluated HCC patients suffered from NASH compared to those from ALD and/or HCV who received curative treatment[42]. Although the postoperative mortality and the recurrence free survival (RFS) did not significantly differ between the two groups, NASH patients had longer OS, compared to ALD and

Table 3 Treatment outcomes and prognosis in patients with non-alcoholic fatty liver disease-related hepatocellular carcinoma *vs* other etiologies-related hepatocellular carcinoma

Ref.	Year/country/type of study	Total HCC patients (underlying disease)	Features of hepatocellular carcinoma (NAFLD *vs* other etiologies)	Treatment	Overall survival (NAFLD *vs* other etiologies)	Recurrence-free survival (NAFLD *vs* other etiologies)
Younossi *et al*[30]	2015/United States/Retrospective	4979 with HCC; 701 NAFLD, 254 AH/BC, 817 ALD, 471 HBV, 2736 HCV	NAFLD: ↑ possibility of unstaged HCC *vs* HCV/HBV	LT	NAFLD: ↓ OS *vs* HCV/HBV (NAFLD: 1-yr mortality risk is 61% *vs* 50% for the HCV/HBV group)	NA
Golabi *et al*[94]	2017/United States/Retrospective	11187 total HCC patients; 1277 NAFLD, 1421 ALD, 586 HBV, 3591 HCV	Among HCC patients treated with SR: 57% had HCV *vs* 17% had NAFLD	LT, SR, TACE	NAFLD: ↓ OS *vs* HCV and/or HBV (HR: 0.82) but ↑ OS *vs* ALD (HR: 1.59)	NA
Piscaglia *et al*[24]	2016/Italy/Prospective	756 total HCC patients; 145 NALFD, 611 HCV	NAFLD: More advanced BCLC HCC stage and more commonly outside the Milan criteria *vs* HCV	LT, SR, PEI, Thermal ablation, TACE, BSC or trials	NAFLD: ↓ 1-yr and 3-yr OS *vs* HCV (1-yr and 3-yr survival; 76.4% and 48.7% in the NAFLD-HCC group and 84.2% and 61.1% in the HCV-HCC respectively) NSD among treatment choices	NA
Hester *et al*[96]	2019/United States/Retrospective	1051 total HCC patients; 92 NASH, 153 ALD, 87 HBV, 719 HCV	NASH and HBV HCC patients: Larger median tumor size *vs* HCV and ALD. NSD in BCLC staging among the groups	LT, SR or ablative techniques, TACE, yttrium 90, or TARE or radiation therapy, systemic therapy	NAFLD: ↓ OS *vs* ALD (HR: 1.92) NSD between NAFLD-HCC and viral-related HCC	NA
Giannini *et al*[97]	2009/Italy/Prospective	471 total HCC patients; 45 CC, 426 HCV	CC: ↑ prevalence of multinodular and diffuse lesions, ↑ size of the largest lesion and advanced classification according to Milano criteria (69% *vs* 41%) *vs* HCV	LT, SR, PEI, RFA, TACE	CC: ↓ OS *vs* HCV	NA
Koh *et al*[34]	2019/Singapore/Prospective	996 total HCC patients; 152 with NAFLD, 844 non-NAFLD	NAFLD: Smaller median tumor size	Total liver resection	NAFLD: ↑ 5-yr and 10-yr OS *vs* non-NAFLD groups (5-yr and 10-yr OS; 70.1% and 49.6% in the NAFLD-HCC group *vs* 60.9% and 41.0% in the non-NAFLD-HCC respectively)	NSD in RFS (P = 0.0931)
Reddy *et al*[42]	2012/United States/Retrospective	303 HCC patients; 52 with NAFLD *vs* 162 HCV/ALD	NASH: NSD in largest tumor size, tumor differentiation and presence of satellite lesions *vs* HCV/ALD	Resection, ablation, and LT	NASH: ↑ 3-yr OS *vs* HCV/ALD (60.9% *vs* 36.2%)	NSD
Benhammou *et al*[98]	2020/United States/Retrospective	454 total HCC patients; 125 NAFLD, 170 HBV, 159 HCV	NAFLD and HCV more likely to be within Milan and UCSF criteria for LT *vs* HBV	LT, SR, RFA, PEI, TACE/Y-90, chemotherapy, BSC	NAFLD: ↑ OS *vs* HBV (HR: 0.35) and HCV (HR: 0.37)	NAFLD: ↑ RFS *vs* HCV (HR: 0.64) and HBV (HR: 0.69)
Viganò *et al*[99]	2015/United States/Retrospective	1563 total HCC patients; 96 HCV, 96 MetS matched	MetS: NSD in satellite nodules and microvascular invasion *vs* HCV	SR, preoperative PVE, TACE	MetS: ↑ OS *vs* HCV (65.6% *vs* 61.4%)	MetS: Trend for ↑ RFS *vs* HCV (37.0% *vs* 27.5%, P = 0.077)
Bengtsson *et al*[40]	2019/Sweden/Retrospective	1562 total HCC patients; 225 NAFLD, 1337 non-NAFLD	NAFLD: NSD in BCLC staging, number of tumors and largest tumor size *vs* non-NAFLD	LT ± RFA or TACE, SR, RFA, TACE, systemic therapy or BSC	NAFLD: NSD in OS *vs* non-NAFLD (HR: 1.04)	NA
Than *et al*[100]	2017/United States/Retrospective	487 total HCC patients; 212 NAFLD, 275 HCV	NAFLD: ↑ tumor size *vs* HCV	TACE, RFA, SR, PEI, sorafenib, LT	NAFLD: NSD *vs* HCV (44% *vs* 56% respectively)	NA

Wakai *et al*[101]	2011/Japan/Retrospective	225 total HCC patients; 17 NAFLD, 61 HBV, 147 HCV	NAFLD: ↑ tumor size *vs* HCV & HBV	SR	NAFLD: ↑ postoperative morbidity and 30-d mortality rates (59% and 12% in NAFLD *vs* 31% and 0.7% in HCV respectively & 28% and 3.3% in HBV respectively)	NAFLD: ↑ RFS *vs* HBV & HCV
Jung *et al*[95]	2021/South Korea/Retrospective	426 total HCC patients; 32 NAFLD, 200 HBV, 194 HBV/NAFLD	NAFLD: ↑ average tumor size *vs* HBV group (4.4 cm *vs* 3.4 cm)	Hepatectomy	Before PSM: NAFLD: ↓ 5-yr OS *vs* HBV (63% *vs* 80%). After PSM, NSD in 5-yr OS rates	NSD in RFS or disease-specific survival before and after PSM
Tokushige *et al* [102]	2010/Japan/Prospective	90 total HCC patients; 34 NASH, 56 HCV	NASH: NSD in tumor size *vs* HCV	SR, RFA, TACE	NASH: NSD in 5-yr survival rate (55.2% in NASH *vs* 50.6% in all HCV)	NSD in 5-yr recurrence rate
Pais *et al*[15]	2017/France/Retrospective	323 total HCC patients; 39 NAFLD, 284 non-NAFLD	NAFLD: ↑ larger tumor size *vs* non-NAFLDNSD in other tumor characteristics	SR, TACE, PVE, PEI, LT	NSD in 2.5 post-LT OS (Mortality: 36% in NAFLD, 48% in ALD, 45% in HCV and 36% in CHB)	NSD
Hernandez-Alejandro *et al* [103]	2012/Canada/Retrospective	81 total HCC patients; 17 NASH, 64 HCV	NASH: ↓ proportion had poorly differentiated HCC *vs* HCV	LT	NA	NASH: trend of ↑ 5-yr RFS (P = 0.11)

HCC: Hepatocellular carcinoma; NAFLD: Non-alcoholic fatty liver disease; AH/BC: Autoimmune hepatitis/Biliary cirrhosis; ALD: Alcohol-related liver disease; HBV: Hepatitis B virus; HCV: Hepatitis C virus; LT: Liver transplantation; OS: Overall survival; NA: Not applicable; SR: Surgical resection; TACE: Transarterial chemoembolization; BCLC: Barcelona-Clinic Liver Cancer; PEI: Percutaneous ethanol injection; BSC: Best supportive care; NSD: No significant difference; RFS: Recurrence-free survival; NASH: Non-alcoholic steatohepatitis; TARE: Transarterial radioembolization; HR: Hazard ratio; CC: Cryptogenic cirrhosis; RFA: Radiofrequency ablation; MetS: Metabolic syndrome; PVE: Portal vein embolization; OR: Odds ratio; UCSF: University of California at San Francisco.

HCV patients, independently of clinical factors and type of the curative treatment they received[42]. In another study, during a median follow-up of 17 mo, NAFLD-HCC patients displayed significantly improved OS and a trend towards increased RFS, compared to both HCV and HBV patients, in a model adjusted for demographic factors, Child-Pugh score and most definite treatment[98]. Notably, in order to assess the afore-mentioned long-term outcomes independently of the LT, authors omitted the LT recipients from all groups[98]. To this end, they showed that the NAFLD-HCC patients still had significantly improved OS rates compared to their HCV counterparts and a trend towards increased survival compared to HBV patients[98]. In 2015 Viganò *et al*[99], matched 96 HCC patients with MetS with 96 HCV-HCC patients who received liver resection during a 12-year study period[99]. Matching was based on age, prevalence of cirrhosis, Child-Pugh class, portal hypertension and HCC characteristics [99]. MetS-HCC patients had significantly better OS and lower recurrence rate compared to HCV-HCC cases whereas in the multivariate analysis MetS-HCC was an independent protective factor for both OS and early recurrence[99].

Finally, several studies have highlighted the similar long-term outcomes regarding NAFLD and non-NAFLD-HCC patients. A Swedish retrospective study revealed that although NAFLD-HCC patients had higher age, higher prevalence of comorbidities and less HCC surveillance, they had similar survival to non-NAFLD-HCC patients, mainly attributed to the poor prognosis of HCC in general[40]. Consistently, Than *et al* [100] compared the outcomes of 212 NAFLD-HCC and 275 HCV-HCC patients who

were referred for LT and showed that the 3-year post-diagnosis OS was similar in the two groups[100] and this finding was confirmed in the subgroup-analysis of patients who eventually received LT[100]. Along this line, Wakai *et al*[101] upon evaluating 317 HCC patients who received hepatic resection, showed that the 5-year post-resection cumulative survival rate did not differ significantly between NAFLD and non-NAFLD groups. Yet, RFS following liver resection was markedly better in the NAFLD group [101]. Additionally, Jung *et al*[95] reviewed the outcomes of NAFLD-HCC and HBV-HCC patients who underwent hepatectomy over a 10-year period. After a median follow up of 74 mo, the latter had superior 5-year OS rates, compared to the former. However, when the authors performed a propensity score matching to minimize the bias of lead time, 5-year OS was similar between the two groups[95]. In a Japanese prospective study, the OS and the RFS after curative treatment of NAFLD-HCC and HCV-HCC patients were evaluated, and both were comparable between the two cohorts[102]. Similarly, Pais *et al*[15] in their retrospective study evaluating a 20-year period confirmed those results[15], while a Canadian study revealed only a trend for better 5-year RFS of NASH-HCC, compared to HCV-HCC patients who underwent LT [103].

However, we should emphasize that due to less intense surveillance of HCC in general, the disease is likely to be diagnosed at advanced stages and therefore even fewer patients are eligible for radical therapy or LT. Thus, the prognostic outcomes of NAFLD-HCC patients compared to non-NAFLD-HCC ones, should be interpreted with cautiousness and accordingly to each clinical study design and type of HCC-treatment that patients received.

Moreover, another factor raising concern for defining the long-term survival outcomes of NAFLD-HCC patients is that those patients seem to have a lower MELD score waiting at LT list compared to their non-NAFLD counterparts, as they tend to have a more preserved liver function. Therefore, they are less likely to receive LT in short-term. This in turn results in longer duration in the waiting list increasing the risk for severe health-related complications and morbidity negatively affecting their survival. Indeed, when Wong *et al*[104] retrospectively analysing UNOS registry data concerning LT waiting list registrations in the United States, demonstrated that NAFLD patients as compared to their HCV or ALD counterparts, were markedly less likely to receive a liver transplant within 90 d and one year after their registration, ending in higher mortality while on the waiting list[104].

SURVEILLANCE

Since NAFLD patients seem to be frequently diagnosed in advanced tumor stages, their surveillance for HCC development represents quite the most challenging issue among professional societies worldwide. As for the cirrhotic-NAFLD patients, since they appear to have an expected HCC incidence of approximately 1.5% *per* year, they should follow the screening guidelines for cirrhotic patients of any cause, consisted of abdominal examination with liver ultrasonography with or without alpha-fetoprotein (AFP) every 6 mo[105] (Figure 2).

Regarding non-cirrhotic NAFLD patients, there is a lack of consensus whether NAFLD patients with F3 fibrosis should undergo screening. The updated recommendation of EASL guidelines suggest that patients with F3-fibrosis might be eligible for HCC surveillance based on an individual risk stratification[106], while the clinical update of the American Gastroenterology Association also recommend the screening for patients with findings indicative of advanced fibrosis (F3), as evaluated by two or more concordant non-invasive fibrosis tests of separate categories[107]. AASLD guidelines recommend HCC surveillance only in cirrhotic (not advanced fibrosis-F3) patients[108]. Finally, the Asian guidelines do not provide specific recommendation for non-cirrhotic NAFLD patients[109] (Figure 2). However, some concerns are raised. Even if a consensus for screening of F3 patients was reached, a large proportion of HCC cases that occur in F0-F2 NAFLD patients would still be missed. Moreover, the diagnosis of F3 fibrosis based on a broad spectrum of non-invasive tests mitigates the utility of screening since HCC risk would not still be the same for all F3-patients, while screening all patients with F3 fibrosis would drastically increase the cost of the surveillance strategy. Moreover, it should be mentioned that even among cirrhotic patients, there are differences in risk for HCC and therefore they should not be aggregated into a single category. Ioannou *et al*[110] developed a predictive model that estimates HCC risk in cirrhotic-NAFLD patients by implicating demographic, clinical and laboratory parameters[110]. Based on this model, patients

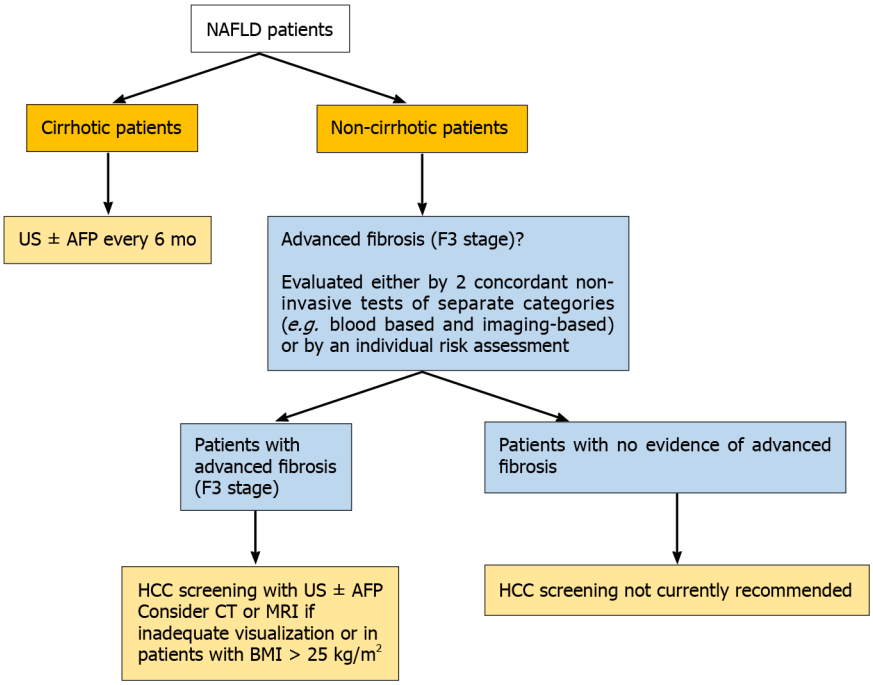

Figure 2 Proposed algorithm for hepatocellular carcinoma surveillance in non-alcoholic fatty liver disease patients based on the latest guidelines. HCC: Hepatocellular carcinoma; NAFLD: Non-alcoholic fatty liver disease; US: Ultrasonography; AFP: Alpha-fetoprotein; CT: Computer tomography; MRI: Magnetic resonance imaging; BMI: Body mass index.

were categorized into low-risk (annual risk: < 1%), medium-risk (annual risk: 1%–3%) and high-risk (annual risk: > 3%), suggesting that individualized screening for HCC is associated with standardized benefit compared with screening of all cirrhotic-NAFLD patients[110,111]. Moreover, the identification of PNPLA3rs738409C>G or other polymorphisms might be of value for screening since it would improve prediction accuracy, but its evaluation in multi-factorial risk models would decrease the cost-effectiveness of patients' surveillance[112]. The future surveillance policies should focus on identification of prognostic factors, consisting of imaging modalities, serum biomarkers and genetic variants, testing for which would need to become cheaper, that will stratify the risk of HCC in both cirrhotic and non-cirrhotic NAFLD patients promoting the cost-effectiveness of the programmes. The afore-mentioned parameters along with well-established traditional risk factors of HCC may be incorporated in future risk-assessment models and could result in more accurate prediction of NAFLD-related HCC and optimized surveillance strategies. A proposed algorithm of NAFLD-related HCC surveillance based on future perspectives is illustrated in Figure 3.

As yet, circulating micro-RNAs (miRNAs) and long non-coding-RNAs have been shown promising results since they were associated with HCC progression in NAFLD patients and may constitute potential non-invasive tools for NAFLD-related HCC screening[113,114]. Several micro-RNAs, such as miR-29 and miR-199, mainly expressed in NASH, are associated with fibrosis progression and HCC development [113]. Furthermore, hydroxy-methylated genes are strongly related to the involvement of chromatin in the progression of HCC and form promising genetic factors for the risk classification of AFP-negative HCC patients[115]. Results derived from animal models examining the progression of HCC in NASH mice, suggested that serum osteopontin and dikkopf-1 could be possible novel biomarkers for the early detection of HCC[116]. Finally, the identification and amplification of circulating tumor-DNA can reveal critical HCC-related genetic mutations and therefore could be used for the screening of HCC patients[117]. However, the incorporation of those prognostic biomarkers in screening programmes would significantly increase the cost with ambiguous results. As until now, they comprise mostly future perspectives rather than clinical point-of-care practice.

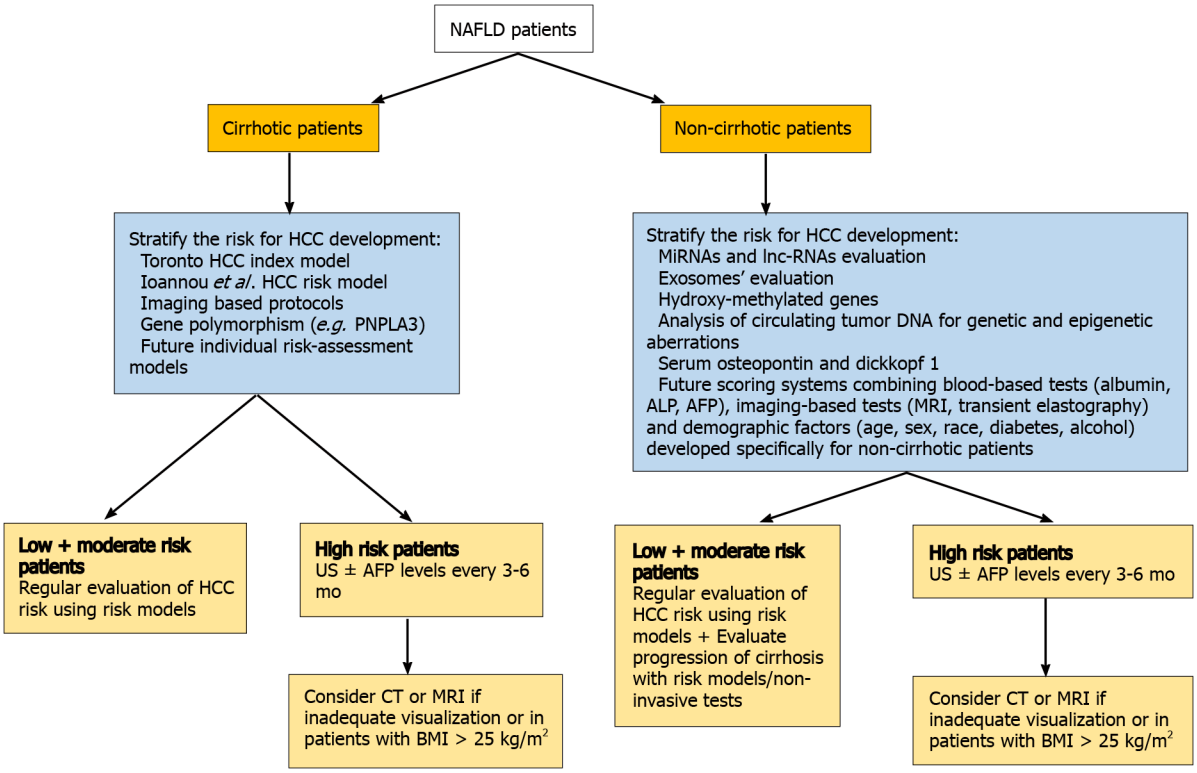

Figure 3 Proposed algorithm for hepatocellular carcinoma surveillance in non-alcoholic fatty liver disease patients based on future perspectives. HCC: Hepatocellular carcinoma; NAFLD: Non-alcoholic fatty liver disease; PNPLA3: Patatin-like phospholipase domain-containing protein 3; MiRNAs: Micro-RNAs; lnc-RNAs: Long non-coding-RNAs; ALP: Alkaline phosphatase; AFP: Alpha-fetoprotein; US: Ultrasonography; CT: Computer tomography; MRI: Magnetic resonance imaging; BMI: Body mass index.

PREVENTION

Although weight loss is considered fundamental for the management of NAFLD, there is no current evidence to directly indicate that weight loss leads to reduction of NAFLD-related HCC. Importantly, a recent large multi-national study with 467336 individuals, demonstrated that physical exercise, defined as performing at least 2 hours of vigorous activity *per* week, can reduce the risk of developing HCC independently of other risk factors of HCC[118]. Moreover, the prevention of obesity and T2DM is considered fundamental for the management of NAFLD patients, since they constitute independent risk factors for HCC development and progression[80,82,111,119]. Noteworthy, a meta-analysis of 19 studies showed that diet rich in vegetables may reduce HCC incidence, while George *et al*[120] suggested that stricter adherence to Mediterranean diet was protective against HCC development[120]. Increased coffee consumption is also associated with decreased risk of NAFLD development and severity progression[121], with two additional coffee cups *per* day to be associated with 35% lower incidence of HCC[122]. However, the exact impact of coffee consumption as a preventive measure against NAFLD and its progression to HCC needs further investigation. Although literature data are controversial regarding the role of light and moderate alcohol use in NAFLD *per se*[89,90], in a study of 195 cirrhotic-NASH patients, Ascha *et al*[86] demonstrated that patients with any alcohol consumption had higher risk of HCC incidence compared to non-drinkers[86]. Consistently, HCC occurrence was more frequent in NAFLD patients with mild alcohol intake (< 20 g/d), especially in those with advanced fibrosis, as compared to abstainers patients[123]. Concerning medication, in a prospective study of 361 NAFLD patients, daily use of aspirin was associated with significant lower odds for NASH and advanced fibrosis, while no relationship between use of non-aspirin nonsteroidal anti-inflammatory drugs and risk for advanced fibrosis was outlined[124]. Furthermore, the administration of statins in cirrhotic patients provides chemo-preventive effects and is associated with the reduction of HCC occurrence, in a dose-dependent manner [125-127]. Interestingly, fluvastatin, compared with other statin interventions, exhibited the most significant effect in the reduction of HCC incidence in cirrhotic patients, while the utility of rosuvastatin against the development of NAFLD-related

HCC was shown in a murine model[127,128]. As far for T2DM pharmacotherapy, dose-response anti-tumorigenic effects of metformin were observed among T2DM patients[129], while in contrast, the administration of sulfonylureas and insulin have been associated with increased risk of HCC[129].

CONCLUSION

In the 21st century, we are in the midst of an epidemic of obesity, T2DM and NAFLD. Consequently, the burden of NAFLD in HCC development is rapidly rising partially explaining the elevated incidence of HCC in both men and women globally. Although the exact pathogenetic mechanisms involved in NAFLD-related HCC onset are still not well-established especially regarding the non-cirrhotic hepatic parenchyma, specific risk factors for HCC concerning demographic, genetic and behavioral parameters have been already identified. Noteworthy, the surveillance of NAFLD-HCC patients is not standard in medical practice and therefore many patients do not undergo screening and that leads to diagnosis of HCC at advanced stages negatively affecting their survival and diminishing the therapeutic options. Concerning systemic treatment for HCC, the latest data[130,131] do not support the hypothesis that the therapeutic decisions should be based on the underlying HCC etiology and therefore, HCC systemic therapy was not in the field of our review. However, noteworthy, in a recent meta-analysis of 3 trials[132-134], authors suggested that immunotherapy might be less efficacious in NASH-HCC patients as compared to their viral-HCC counterparts, presumably owing to the NASH-provoked aberrant T-cells activation and subsequently flawed immune surveillance[135]. Yet, more robust evidence are needed for therapeutic decision making. Although, lifestyle modifications, such as stricter adherence to Mediterranean diet and medication namely metformin are thought to contribute to the primary prevention of NAFLD-related HCC, the appropriate strategy would be the identification of at-risk patients *via* a relatively simple score including demographic and laboratory/imaging parameters. Implementation of risk stratification programmes and high awareness of the burden of NAFLD should be the primary goals for medical clinical specialties and health authorities worldwide.

ACKNOWLEDGEMENTS

We are grateful to Dr. Panagiotis Lembessis for the language polishing of the manuscript.

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World Journal of Gastroenterology

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World J Gastroenterol 2022 January 21; 28(3): 332-347

DOI: 10.3748/wjg.v28.i3.332

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

MINIREVIEWS

Endoscopic ultrasound role in pancreatic adenocarcinoma treatment: A review focusing on technical success, safety and efficacy

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Author contributions: Khoury T and Sbeit W contributed to study design and concept; Napoléon B contributed to critical revision of the manuscript; all authors contributed to data analysis, writing the initial draft and approved the final version to be published.

Conflict-of-interest statement: The authors declare no conflict of interest.

Country/Territory of origin: Israel

Specialty type: Gastroenterology and hepatology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

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Abstract

The impressive technological advances in recent years have rapidly translated into the shift of endoscopic ultrasound (EUS) from diagnostic modality into an interventional and therapeutic tool. Despite the great advance in its diagnosis, the majority of pancreatic adenocarcinoma cases are inoperable when diagnosed, thus demanding alternative optional therapies. EUS has emerged as an easy, minimally invasive modality targeting this carcinoma with different interventions that have been reported recently. In this review we summarize the evolving role of interventional therapeutic EUS in pancreatic adenocarcinoma management.

Key Words: Endoscopic ultrasound; Pancreas; Cancer; Management; Palliative

Core Tip: The prognosis of pancreatic adenocarcinoma is poor in advanced stages. Several studies were conducted recently to assess the effect of different treatment options provided through endoscopic ultrasound (EUS). We present a comprehensive review on the role of EUS in unresectable pancreatic adenocarcinoma treatment while exploring its effect on survival and palliation. We found that EUS-guided intervention is feasible with excellent technical success, limited adverse events, a beneficial effect on cancer-associated pain and an as-yet unknown effect on survival. For EUS-assisted therapies there are still many unknowns and unanswered questions, prompting the need for additional prospective randomized controlled studies comparing the different

Received: September 28, 2021
Peer-review started: September 28, 2021
First decision: November 18, 2021
Revised: November 22, 2021
Accepted: January 6, 2022
Article in press: January 6, 2022
Published online: January 21, 2022

P-Reviewer: Jin ZD, Nakahodo J
S-Editor: Fan JR
L-Editor: Filipodia
P-Editor: Fan JR

treatment approaches combined with chemo +/- radiotherapy with respect to success, efficacy, safety and survival.

Citation: Sbeit W, Napoléon B, Khoury T. Endoscopic ultrasound role in pancreatic adenocarcinoma treatment: A review focusing on technical success, safety and efficacy. *World J Gastroenterol* 2022; 28(3): 332-347
URL: https://www.wjgnet.com/1007-9327/full/v28/i3/332.htm
DOI: https://dx.doi.org/10.3748/wjg.v28.i3.332

INTRODUCTION

Pancreatic adenocarcinoma is the seventh leading cause of cancer death worldwide with poor prognosis according to the 2020 GLOBOCAN cancer estimates[1]. About half of patients are diagnosed with metastatic disease and 30% with locally advanced disease and are deprived from the only potential cure of surgical intervention[2]. The median overall survival for stages IV and III is of 2-3 and 7-11 mo, respectively[3]. As a result, those patients are usually offered supportive care, palliative chemotherapy and radiology, and palliative surgical interventions. Endoscopic ultrasound (EUS), first introduced about 40 years ago as a diagnostic tool, has quickly gained popularity as an interventional therapeutic tool in a broad range of gastrointestinal, pancreato-biliary and liver diseases due to its high spatial resolution. There are several characteristics of EUS that improve its utility as an interventional therapeutic instrument. The first and most crucial property is its high spatial resolution and the proximity of its transducer to the target lesion, allowing it to access small lesions while avoiding intervening structures, blood vessels and air[4]. The second advantage lies in its minimal invasiveness and high safety profile in targeting pancreatic lesions; these have advanced this modality over interventional radiology and surgery in diverse pancreatic tumor treatment applications[5]. The third advantage is its ability to obtain contrast-enhancement images which seems to improve diagnostic performance in pancreatic masses[2]. The final benefit is the technical advancement in developing devices designed specifically to allow minimally invasive therapeutic interventions[6]. An increasing number of articles reporting these new EUS applications in pancreatic adenocarcinoma have been published, including EUS guided thermal ablation, ethanol ablation, delivery of antitumor agents, brachytherapy, fiducial marker placement (FMP), and EUS-guided celiac plexus neurolysis/block (CPN/B). In this review we summarize the literature dealing with interventional therapeutic EUS in pancreatic adenocarcinoma, aiming to present an updated comprehensive review on this topic.

LITERATURE SEARCH

A search for studies published before August 2021 was performed in the PubMed databases with the keywords EUS or endoscopic ultrasound and any of the following: Carcinoma or adenocarcinoma of pancreas, pancreatic tumor, treatment or therapeutic, intervention, ablation, injection, brachytherapy, fiducial markers and CPN. The search was restricted to articles in the English language and included prospective, retrospective, case series and randomized controlled studies. Review articles and case reports were not included. Subsequently, we generated a state-of-the-art comprehensive review by summarizing the most updated data on EUS-guided intervention published in the last several years and focusing on feasibility, technical success, safety and effect on overall survival and palliation when the data were available.

EUS-GUIDED INTRA-TUMORAL INJECTIONS

Intra-tumoral EUS fine needle injection (EUS-FNI), is a relatively new treat-to-target modality aiming to deliver and potentially achieve high intra-tumor drug concentration while minimizing systemic exposure and toxicity from those drugs[7,8]. This

method allows tumor reduction prior to surgery or serves as a palliative treatment in unresectable tumors with mass effect including obstructive symptoms[8]. EUS-FNI enables performance of several therapeutic interventions including chemotherapy, immunotherapy, gene therapy and intra-tumoral implantation. Table 1 demonstrates all studies of EUS-guided intra-tumoral injections.

CHEMOTHERAPY

A prospective study from Mayo Clinic evaluated EUS-FNI of gemcitabine in 36 patients (long-term data were available in 28 patients) with unresectable pancreatic adenocarcinoma (3 patients with stage II, 20 with stage III and 13 with stage IV). They reported no adverse events, partial response in 25% of patients, stable disease in 57% of patients and down-staging in 20% of stage III patients who underwent surgical resection, with a median of an overall survival of 10.4 mo (95% confidence interval, 2.7-68), and an overall survival of 78%, 44%, and 3% at 6 mo, 12 mo and 5 years, respectively, leading the authors to conclude that this treatment option is feasible, safe, and potentially effective[9].

IMMUNOTHERAPY

Intra-tumoral immunotherapy, including mixed lymphocyte culture and immature dendritic cells, have the ability to induce a tumor-specific immune response which can be effective, not only locally but also on metastatic lesions[7]. The first clinical trial of immunotherapy was published about 20 years ago and enrolled 8 patients with unresectable pancreatic adenocarcinoma who were treated by EUS-FNI of mixed lymphocyte culture (cytoimplant). They showed this treatment option to be feasible without procedure-related complications and with no substantial toxicity. Notably, the median overall survival was 13.2 mo, with tumor response ranging from 'minor' until 'no change'; however, there were no cases of significant or complete tumor response [10]. Later, Irisawa *et al*[11] reported their experience with seven patients suffering from stage IV gemcitabine non-responsive pancreatic adenocarcinoma who underwent EUS-FNI of immature dendritic cells with radiation therapy administered first in five patients. They showed clinical response in three of the patients with no procedure-related adverse event nor dendritic cell-related toxicity, and with an overall median survival rate of 9.9 mo[11]. Another study from Japan evaluated the feasibility, safety and histological change of preoperative EUS fine-needle injection of immature dendritic cells with OK-432 (immune-potentiating agent) in pancreatic cancer patients. In their study, nine patients were enrolled and compared to a group of 15 patients who were operated without dendritic cell injection. They reported no adverse reaction following injection in the nine patients except for one with transient fever, and no significant difference in postoperative complication incidence between both groups or in the overall median survival. Interestingly, two patients in the injection group survived for more than 5 years without disease recurrence. Analysis of resected specimens in the injection group showed that CD83 + cells significantly accumulated in the regional lymph nodes, as well as Foxp3 + cells in the regional and distant lymph nodes[12].

GENE THERAPY

Gene therapy takes advantage of the preference of oncolytic attenuated adenovirus [ONYX-015 (Onyx Pharmaceuticals, United States)] to selectively replicate in malignant cells, leading to their lysis and death[13]. The first study was performed by Hecht *et al*[14] who enrolled 21 patients with locally advanced pancreatic adenocarcinoma in a trial of intra-tumoral ONYX-015 injection *via* EUS in combination with gemcitabine. They demonstrated the feasibility, safety and tolerability of this treatment modality when EUS-FNI was performed through a trans-gastric route with prophylactic antibiotic. However, no convincing evidence of efficacy was shown as only two patients showed partial regression, two showed minor response, and six had stable disease, while 11 had progressive disease, with a median overall survival of 7.5 mo [14]. Furthermore, a subsequent study by Senzer *et al*[15] demonstrated the safety and efficacy of intra-tumoral injection of TNFerade (GenVec Inc, United States), an

Table 1 Human studies reporting endoscopic ultrasound-guided intra-tumoral injection therapies

Ref.	Study design	Cancer stage	EUS-guided intervention	Patients No.	Technical success (%)	Median overall survival (mo)	Pain palliation	Serious adverse events, *n*
Levy *et al*[9], 2017	Prospective	II (*n* = 3); III (*n* = 20); IV (*n* = 13)	Chemotherapy	36	100	10.4	Not reported	0
Chang *et al* [10], 2000	Prospective	II (*n* = 4); III (*n* = 3); IV (*n* = 1)	Immunotherapy	8	100	13.2	Not reported	0
Irisawa *et al* [11], 2007	Prospective	IV (*n* = 7)	Immunotherapy	7	100	9.9	Not reported	0
Endo *et al*[12], 2012	Prospective	II (*n* = 1); III (*n* = 5); IV (*n* = 3)	Immunotherapy	9	100	18	Not reported	3[1]
Buscail *et al* [23], 2015	Prospective	III (*n* = 13); IV (*n* = 9)	Gene therapy	22	100	12.6	Not reported	0
Hecht *et al* [14], 2003	Prospective	III (*n* = 9); IV (*n* = 21)	Gene therapy	21	100	7.5	Not reported	4[2]
Hecht *et al* [16], 2012	Prospective	III (*n* = 27)	Gene therapy	27/50	100	9.9	Not reported	40[3]
Herman *et al* [17], 2013	Prospective	III (*n* = 95)	Gene therapy	95/187	100	11.5	Not reported	48[4]
Hanna *et al* [18], 2012	Prospective	Unresectable	Gene therapy	6	100	6	Not reported	1[5]
Hirooka *et al* [20], 2018	Prospective	III (*n* = 9)	Gene therapy	9	100	15.5	Not reported	2[6]
Nishimura *et al*[22], 2018	Prospective	III (*n* = 5); IV (*n* = 1)	Gene therapy	6	100	5.8	Not reported	0
Golan *et al* [25], 2015	Prospective	III (*n* = 15)	Intra-tumoral implantation	15	100	15.1	Not reported	4[7]

[1]Pancreatic fistula (2 patients) and superior mesenteric artery pseudoaneurysm (1 patient).
[2]Sepsis (2 patients), duodenal perforations (2 patients).
[3]The authors did not state whether these adverse events were in the endoscopic ultrasound (EUS) group or in the percutaneous group: Gastrointestinal bleeding (6 patients), deep vein thrombosis (6 patients), pulmonary embolism (2 patients), pancreatitis (2 patients), cholecystitis (1 patient), biliary obstruction (8 patients), cholangitis (6 patients), hypotension (2 patients), bradycardia (1 patient), supraventricular tachycardia (1 patient), splenic artery thrombosis (1 patient), intestinal ischemia (1 patient), staphylococcus infection (1 patient), cerebrovascular accident (1 patient), cardio-pulmonary arrest (1 patient).
[4]The authors did not state what are the serious adverse events and whether these adverse events were in the EUS group or in the percutaneous group.
[5]Hypoglycemia (1 patient).
[6]Perforation of duodenum (1 patient) and hepatic dysfunction 1 patient), but these events were considered not to be related.
[7]Colonic obstruction (1 patient), pancreatitis (1 patient), cholangitis (1 patient), renal failure (1 patient).

adenovirus vector with replication deficiency that carries the human tumor necrosis factor-alpha gene regulated by a radiation-inducible promoter, followed by radiation has been demonstrated in a phase I clinical trial of 30 patients with solid tumors, 21 of 30 patients (70%) demonstrated objective tumor response (five complete, nine partial, and seven minimal responses), with only mild toxicities reported as the most common adverse event, including fever (22%), injection site pain (19%) and chills (19%)[15]. A phase I/II non-randomized study enrolled 50 patients for intra-tumoral TNFerade treatment with 5-fluorouracil and radiotherapy for locally advanced pancreatic cancer (27 patients were administered under EUS guidance and 23 patients through the percutaneous route). Their results showed promise with intra-tumoral TNFerade injection, with an overall median survival of 9.9 mo, and median time-to-tumor progression of 3.6 mo, as one patient had complete response, three had partial response, and twelve had stable disease, while 19 patients had progressive disease. Notably, there was a high safety signal in this study, as 40 serious adverse events were recorded, however we were unable to extract whether these adverse events were in the EUS group or in the percutaneous group, as this information was not supplied by the authors[16]. In a randomized phase III multi-institutional study enrolling 304 patients, 187 were treated with standard of care and TNFerade (95 patients under EUS-

guidance and 91 percutaneously) *vs* 117 who received only standard of care therapy. Although the method was shown to be safe, it did not lead to prolonged survival, as the median overall survival was 10 mo in both the 'standard of care and TNFerade' and the 'standard of care alone' groups. Notably in that study, serious related adverse events occurred in 48 patients (25.7%) of the 'standard of care and TNFerade' group, as compared to 20 patients (16.7%) in the 'standard of care' group ($P = 0.13$); however, the serious adverse events were not detailed and the authors did not report whether those adverse events occurred in the EUS or the percutaneous sub-group of the 'standard of care and TNFerade' group[17]. BC-819 is a double-stranded DNA plasmid designated to target the expression of diphtheria-toxin gene under the control of H19 regulatory sequences, and thus have the potential to treat cancer with H19 overexpression. The pharmacokinetics, tolerability and safety and preliminary efficacy of intra-tumoral-injected BC-19 were assessed in a phase 1/2a study of nine patients with unresectable pancreatic adenocarcinoma. The authors reported no increase in tumor size 4 wk after receipt of first treatment, down staging and conversion into resectable cancer in two patients and partial response in three patients after 3 mo. Remarkably, only one spontaneously-resolving asymptomatic lipase elevation considered to be an adverse event, occurred. BC-819 combined with systemic chemotherapy may have additive therapeutic benefit in these patients[18]. Another oncolytic virus is HF10 that enjoys the unique property of being a spontaneous mutation product of herpes simplex virus-1 without artificial modification. It has a high affinity to tumor cells and high replication leading to antitumor immune response[19]. A phase I clinical trial of EUS-guided intra-tumoral injection of HF10 in combination with erlotinib and gemcitabine in 10 patients with unresectable locally-advanced pancreatic cancer reported three partial responses, four stable disease and two progressive diseases in the nine subjects who completed the treatment. However, five patients showed Grade III myelosuppression and two patients developed serious adverse events (perforation of duodenum, hepatic dysfunction), though these events were considered to be unrelated to HF10. Two patients underwent R0 surgical resection after down staging. The median progression-free survival was 6.3 mo and the overall survival 15.5 mo[20]. The effect of the synthetic double stranded RNA oligonucleotide, STNM01, known to selectively inhibit the expression of carbohydrate sulfotransferase-15 (CHST-15)[21], was explored by Nishimura *et al*[22], who injected STNM01 intra-tumorally with EUS-guidance in six patients with unresectable pancreatic cancer. They reported tumor necrosis in biopsy in four patients and significant reduction of CHST15 in two patients, with an overall survival of 15 mo in these two patients, but only 5.7 mo in the other four patients. The authors concluded that EUS-FNI of STNM01 in these patients is safe and feasible[22]. A previous interesting study with 22 patients aimed to assess the effect of CYL-02, a non-viral gene therapy targeted to sensitize pancreatic cells to chemotherapy, reported promising results. Nine patients showed stable disease up to 6 mo following treatment and two of these patients experienced long-term survival, with a median overall survival of 12.6 mo, and without serious adverse events[23].

INTRA-TUMORAL IMPLANTATION

Zorde Khvalevsky *et al*[24] developed a local prolonged siRNA delivery system (Local Drug EluteR, LODER) releasing siRNA against the mutated KRAS (siG12D LODER), enabling siRNA protection from degradation and prolonged periods of intra-tumoral slow release with proved therapeutic efficacy[24]. The tolerability, efficacy and safety of EUS-guided intra-tumoral injection of miniature biodegradable implant siG12D-LODER, releasing a specific silencing RNA against K-RAS mutations in combination with chemotherapy for locally advanced pancreatic cancer patients was shown in a study by Golan *et al*[25]. Their open-label Phase 1/2a study included 15 patients; of the 12 patients analyzed by computed tomography (CT) scans, 10 demonstrated stable disease and two showed partial response. Seven patients had a decrease in tumor marker CA19-9. The median overall survival was 15.12 mo. Serious adverse events were reported in four patients[25].

COMBINATION'S INJECTION

A recent phase 1 study by Lee *et al*[26] evaluating the safety and tolerability of Ad5-yCD/mutTK(SR39)rep-ADP (Ad5-DS), a replication-competent adenovirus-mediated double-suicide gene therapy in combination with gemcitabine, demonstrated the good

tolerability and safety of this combination[26]. Five of their nine patients with inoperable locally-advanced pancreatic cancer treated with the combination of intravenous gemcitabine and EUS-FNI of dendritic cell followed by intravenous infusion of lymphokine-activated killer cells, showed response without treatment-related severe adverse events[27]. Another study evaluated the feasibility, safety and efficacy of EUS-FNI of zoledronate-pulsed dendritic cell combined with intravenous administration of αβT cells and gemcitabine in 15 patients with locally-advanced pancreatic adenocarcinoma. Most of these patients had clinical response and seven had stable disease; the authors concluded that this combination may have a therapeutic benefit. Adverse events were reported in four patients, two of which were related to gemcitabine[28].

EUS-GUIDED ABLATION THERAPIES

Dedicated ablation devices are designed to perform specific ablative procedures in patients with inoperable pancreatic cancer, or who are at high surgical risk or refuse surgery. The procedures include ethanol ablation, thermal ablation including hybrid cryothermal ablation, radiofrequency ablation (RFA), Photodynamic ablation (PDT) and laser ablation[6]. Table 2 shows all studies of EUS-guided ablation therapies.

ETHANOL ABLATION

Ethanol is an attractive ablative agent due to its wide availability, low cost and efficacy. Once injected it causes rapid coagulation necrosis resulting from protein denaturation, cell membrane lysis and vascular occlusion[29]. Its superiority over the percutaneous route resides in its proximity to the pancreas, allowing precise localization and measurement of the lesion with real-time imaging, thus minimizing damage to surrounding normal tissue[30]. To date, we could identify only one study that reported the effect of EUS-guided ethanol injection in pancreatic adenocarcinoma: Facciorusso *et al*[31] evaluated pain management in 123 patients with pancreatic adenocarcinoma, as well as the treatment's effect on overall survival. That study compared the efficacy and safety of EUS-guided tumor ethanol ablation in combination with CPN (65 patients) *vs* CPN alone (58 patients). The combination therapy was shown to be significantly superior to CPN alone in terms of pain relief (P = 0.005) and complete pain response (P = 0.003), with additional survival benefit (8.3 mo *vs* 6.5 mo, respectively). The median duration of pain relief lasted for 18 d (range 13-20) in the combined group, as compared to 10 d (range 7-14) in the CPN group (P = 0.004)[31].

RFA

The high temperature, ranging between 60-100 °C induced by RFA results in irreversible cellular damage, apoptosis and coagulative necrosis[32]. Additionally, it is believed that RFA induces immunomodulatory activity, with anticancer effect[33]. EUS-guided RFA is a minimally invasive, feasible, easy and safe ablative modality that constitutes the ablative modality of choice for several solid tumors[34]. Several small-case series recently assessed this modality in pancreatic cancer. Three feasibility studies were performed in this field; the first was by Song *et al*[35] in which six patients with unresectable pancreatic ductal adenocarcinoma were enrolled to assess feasibility and safety of this modality. This study demonstrated an ablation area within the tumor by contrast- enhanced EUS, with no major side effects (two patients suffered from mild abdominal pain) and with complete technical success[35]. The second study by Crinò *et al*[36] evaluated the technical success, feasibility and safety of EUS-guided RFA in eight patients with pancreatic adenocarcinoma and one patient with renal cell metastasis; they reported feasibility in eight patients, with no major side effects. One- and 30-d' CT demonstrated necrosis of about 30% of the tumor. Three patients reported mild abdominal pain. One of the nine patients was excluded due to a large necrotic portion[36]. The third study, by Scopelliti *et al*[37] enrolled 10 patients with pancreatic ductal adenocarcinoma, and reported success in all patients, with no major adverse events, and with scan-documented area of necrosis within tumor at 30 d post-ablation[37]. A study of 30 patients examined whether SMAD4 status affects post-RFA

Table 2 Human studies reporting endoscopic ultrasound-guided ablation therapies

Ref.	Study design	Cancer stage	EUS-guided intervention	Patients No.	Technical success (%)	Median overall survival (mo)	Pain palliation (patients %)	Serious adverse events, *n*
Facciorusso *et al* [31], 2017	Prospective	III (*n* = 50); IV (*n* = 15)	Ethanol ablation	65	100	8.3	90.7 at week 2	0
Song *et al*[35], 2016	Prospective	III (*n* = 4); IV (*n* = 2)	RFA	6	100	-[1]	Not reported	0
Crinò *et al*[36], 2018	Prospective	III (*n* = 8)	RFA	8	100	-[1]	Not reported	0
Scopelliti *et al* [37], 2018	Prospective	III (*n* = 10)	RFA	10	100	-[1]	Not reported	0
Paiella *et al*[38], 2018	Retrospective	Not reported	RFA	30	100	15	Not reported	0
Bang *et al*[39], 2019	Prospective	II (*n* = 2); III (*n* = 3); IV (*n* = 7)	RFA	12	100	Not reported	Significant	0
Arcidiacono *et al* [41], 2012	Prospective	III (*n* = 22)	HCA	22	72.8	6	Not reported	1[2]
DeWitt *et al*[45], 2019	Prospective	III (*n* = 12)	PDA	12	100	11.5	Not reported	0
Di Matteo *et al* [46], 2018	Prospective	III (*n* = 9)	Laser ablation	9	100	7.4	Not reported	0

[1]Feasibility studies not aimed to assess impact on overall survival.
[2]Minor bleeding in duodenal lumen successfully stopped by hemoclips (1 patient). RFA: Radiofrequency ablation; HCA: Hybrid cryothermal ablation; PDA: Photodynamic ablation; EUS: Endoscopic ultrasound.

disease-specific survival in patients with locally advanced pancreatic adenocarcinoma. Results showed that patients with wild-type SMAD4 survived significantly longer than patients with mutant type SMAD4 (22 mo *vs* 12 mo, respectively) with an overall estimated post-RFA disease-specific survival of 15 mo, probably indicating that this gene may help in selecting patients for RFA[38]. Moreover, a recent study by Bang *et al* [39] assessed the role of EUS-guided RFA for pain relief in pancreatic cancer as compared to EUS-guided CPN, and revealed that the EUS-guided RFA was associated with significant improvement in pain associated with pancreatic cancer ($P < 0.05$), in addition to less-severe gastrointestinal symptoms, with better quality of life and emotional functioning[39].

HYBRID CRYOTHERMAL ABLATION

Using a flexible hybrid bipolar cryotherm probe, it is possible to combine radiofrequency with cryotechnology. Cryo is believed to induce a systemic inflammatory response with an antitumor response in addition to the thermal ablation induced by RFA[40]. Only one prospective clinical trial of this type was conducted in 22 patients with locally-advanced pancreatic cancer. Treating them with this hybrid intervention was technically successful in 72.8% of patients, with median post-ablation survival of 6 mo. The few late complications were mainly related to tumor progression, and the single immediate complication of duodenal bleeding was resolved by placing of hemoclips[41]. However, more data are needed to assess this treatment modality.

PDT

PDT is a tumor-specific ablative treatment performed through a combination of photosensitizing drug administration with EUS-guided light irradiation, resulting in cell death by generating oxygen free radicals[42,43]. EUS-guided PDT was first published by Choi *et al*[44], who reported the first preliminary feasibility data for EUS-PDT in patients suffering from locally advanced pancreaticobiliary malignancies. They

enrolled four patients, the first with pancreatic tail carcinoma, the second with distal CBD carcinoma and two patients with carcinoma of the caudate lobe of the liver. The treatment was effective and safe, as it induced a necrotic area of 4 cm^3 without side effects. Notably, disease remained stable for a mean of 5 mo[44]. Recently, a prospective, dose-escalation phase 1 study of 12 patients with locally-advanced pancreatic cancer, treated with EUS-PDT and subsequent gemcitabine therapy 25 d later, showed tumor necrosis in 50% of patients, median progression-free and overall survival were 2.6 and 11.5 mo, respectively. Two patients were operated on, one of them had a complete response and the other one had a residual 2-mm tumor. Notably, there were eight serious adverse events but none related to EUS or EUS-PDT[45]. More data are needed to assess EUS-guided PDT on survival and palliation.

LASER ABLATION

To date, EUS-guided laser ablation has been reported by a single clinical human study that enrolled nine patients with unresectable pancreatic ductal adenocarcinoma who were unresponsive to previous chemotherapy. These patients were treated by laser ablation suing neodymium-yttrium aluminum garnet (Nd:YAG) laser light with different power settings, by flexible fiber, introduced through 22-gauge fine needle aspiration. The coagulative necrotic ablation area was demonstrated by CT scans at 24 h, 7 and 30 d, and was shown to be optimal with power setting of 4 W/1000J with the largest ablation area without adverse events. The median overall survival was 7.4 mo [46]. However, no data regarding palliative effect was reported, thus more data are warranted.

EUS-FMP

Chemoradiation is offered as adjuvant or neoadjuvant to patients with pancreatic adenocarcinoma; however, one of the major challenges with radiation is the proximity of the pancreas to several vital organs. Intensity-modulated radiation therapy was shown to reduce radiation-induced toxicity in these organs in patients with pancreatic and ampullary cancers[47]. Intra-tumoral FMP serves as a landmark enabling accurate radiation targeting of the tumor with minimal harm to neighboring structures. To date, only several feasibility studies were reported addressing safety and technical success, without reporting the effect on overall survival. The first report of EUS-FMP was published in 2006 by Pishvaian *et al*[48] who successfully placed fiducial markers in six of seven pancreatic cancer patients, with no observed complications[48]. After that, several feasibility studies on FMP under EUS-guidance were reported, showing this to be an easy and safe modality with excellent technical success, enabling accurate radiation targeting and without procedure related adverse events in patients with pancreatic cancer[49-54]. As mentioned earlier, to date, the studies on EUS-guided FMP have reported only technical success and adverse events, with no data on survival and palliative benefit, necessitating further studies to assess their therapeutic effect (Table 3).

EUS-GUIDED BRACHYTHERAPY

EUS-guided brachytherapy is defined as the implantation of radioactive seeds near the pancreatic tumorous tissue, followed by exposure of the seeds to steady emissions of gamma rays which lead to localized ablative effect. About two decades ago, Sun *et al* [55] showed that EUS-guided radioactive seeds into pancreatic tissue in a porcine model is a feasible and safe modality for brachytherapy[55]. The favored radioactive seeds in brachytherapy of the rapidly growing pancreatic cancer are iodine-125 due to their long halftime of 59.7 d, which is appropriate in targeting such rapidly-growing tumors. Importantly, the dose rate of these radioactive seeds is low and their penetration depth does not exceed 1.7 cm, thus minimizing radiation exposure and injury to the neighboring organs[56]. Only a few human studies have been conducted with EUS-guided brachytherapy for pancreatic adenocarcinoma. Sun *et al*[57] reported eight patients with locally advanced pancreatic adenocarcinoma who underwent EUS-guided brachytherapy and showed a favorable effect of this modality on pain severity which was ameliorated in four of the eight patients. The pain decrease lasted for 3.5

Table 3 Human studies reporting endoscopic ultrasound-guided fiducial markers placement, -brachytherapy and -celiac plexus neurolysis

Ref.	Study design	Cancer stage	EUS-guided intervention	Patients No.	Technical success (%)	Median overall survival (mo)	Pain palliation (patients %)	Serious adverse events, *n*
Pishvaian *et al* [48], 2006	Prospective	Unresectable	FMP	7	85.7	-[1]	Not reported	0
Choi *et al*[49], 2014	Prospective	Unresectable	FMP	29	100	-[1]	Not reported	1[2]
Varadarajulu *et al*[50], 2010	Prospective	III (*n* = 9)	FMP	9	100	Not reported	Not reported	0
Park *et al*[51], 2010	Prospective	III (*n* = 57)	FMP	57	94	-[1]	Not reported	0
Sanders *et al* [52], 2010	Prospective	III (*n* = 36); Recurrent (*n* = 15)	FMP	51	90	-[1]	Not reported	1[2]
Dávila *et al*[53], 2014	Prospective	II (*n* = 1); III (*n* = 22)	FMP	23	100	-[1]	Not reported	0
Khashab *et al* [54], 2012	Retrospective	III (*n* = 39)	FMP	39	100	-[1]	Not reported	0
Sun *et al*[57], 2012	Prospective	III (*n* = 8)	Brachytherapy	8	100	8.3	50 at week 33	0
Sun *et al*[58], 2006	Prospective	III (*n* = 8); IV (*n* = 7)	Brachytherapy	15	100	10.6	30 at week 42	3[3]
Jin *et al*[59], 2008	Prospective	II (*n* = 4); III (*n* = 10); IV (*n* = 8)	Brachytherapy	22	100	9	81.8 at week 1	0
Sun *et al*[60], 2017	Retrospective	III (*n* = 18); IV (*n* = 24)	Brachytherapy	42	100	9	Not reported	0
Wiersema *et al* [62], 1996	Prospective	Unresectable	CPN	29	100	Not reported	86 at week 2; 84 at week 4; 79 at week 8; 88 at week 12	0
Levy *et al*[64], 2019	Prospective	II (*n* = 2); III (*n* = 27); IV (*n* = 31)	CPN	60	100	10.46	40.4 at week 12	0
Seicean *et al* [65], 2013	Prospective	Unresectable	CPN	32	100	Not reported	75 at week 2	0
Facciorusso *et al* [31], 2017	Prospective	III (*n* = 48); IV (*n* = 10)	CPN	58	100	6.5	70.6 at week 2	0

[1]Feasibility studies not aimed to assess impact on overall survival.
[2]Pancreatitis supportively treated (1 patient).
[3]Pancreatitis complicated with pseudocyst formation (3 patients).
FMP: Fiducial markers placements; CPN: Celiac plexus neurolysis; EUS: Endoscopic ultrasound.

mo and the patients had a median overall survival time of 8.3 mo with no procedure- or treatment-related adverse events[57]. Another study by Sun *et al*[58] reported this treatment modality in 15 patients, with 5 of 15 patients (33.3%) experiencing clinical benefit as assessed by pain reduction and improved Karnofsky performance status score, with a median time-to-achieve clinical benefit of 2.2 mo. Notably, the median overall survival was 10.6 mo, with only three cases of serious complications of pancreatitis complicated with pseudocysts, and no life-threatening adverse events[58]. Similar results were reported by Jin *et al*[59] in 22 patients who showed partial remission in 13.6% of the patients and stable disease in 45.5% during a 4-wk period. Cancer-related pain improved in 18 patients (81.8%) at 1 wk after the intervention, with an estimated median overall survival of 9 mo and no treatment-related adverse events[59]. Finally, the most recent study of this modality performed by Sun *et al*[60] was in 2017 and included 42 patients; once again the research group demonstrated its safety and efficacy, a median overall survival of 9 mo, and no serious adverse events reported[60] (Table 3).

Table 4 Summary of efficacy and safety of endoscopic ultrasound-guided angio-therapy procedures

Procedure	Intra-tumoral injection therapies	Ablation therapies	Fiducial markers placement, brachytherapy and celiac plexus neurolysis
Technical success	High	High	High
Safety (complications)	Uncertain[1]	Minor	Minor
Efficacy			
Survival	Modest	None	None
Palliation	Not reported	Encouraging	High
Mortality	None	None	None

[1]The two studies that had the highest adverse events rate did not state whether they were in the endoscopic ultrasound or in the percutaneous group. See Table 1.

EUS-CPN/B

Abdominal and back pain is a common complaint in pancreatic adenocarcinoma, occurring in about 80% of patients, and it is severe in the majority of patients[61]. Because most patients are diagnosed at an advanced stage, the treatment is mostly palliative, including pain control. The WHO recommends a step-up approach for the control of pancreatic cancer pain, beginning with non-opioid analgesics and progressing to opioid analgesics with increasing dose according to need. Unresponsive patients, those with intolerable side effects, may be candidates for EUS-CPN/B (October 14, 2008. WHO Steering Group on Pain Guidelines). The first description of EUS-CPN/B was by Wiersema *et al*[62] who reported the first human study on EUS-guided brachytherapy in 1996, injection of bupivacaine and 98% dehydrated absolute alcohol in 29 patients with pancreatic adenocarcinoma. Pain score improved in 86%, 84%, 79% and 88% at weeks 2, 4, 8 and 12 post intervention, respectively[62]. Consequently, this therapy rapidly gained popularity as safe and minimally-invasive with the advantage of real-time imaging of blood vessels, compared to the percutaneous route. CPN/B is achieved by alcohol or phenol injection into or around the celiac plexus/ganglion, resulting in its permanent chemical ablation, while CPB is achieved by injecting a corticosteroid in combination with long-acting anesthetic, thus inhibiting pain transmission to the brain[63]. A recent study by Levy *et al*[64] reported the efficacy of EUS-guided CPN/B on the pain score of 60 patients with a pain response rate in 40.4% at 12 wk after intervention, and with an overall survival rate of 10.46 mo[64]. Similarly, another recent study by Facciorusso *et al*[31] reported efficacy in 58 patients, among them 41 patients (70.6%) who achieved pain relief within a median time of 5 d and median pain duration relief of 10 wk, with an overall survival rate of 6.5 mo[31]. The beneficial effect of this modality was shown in a previous study by Seicean *et al*[65] who reported significant pain improvement in 24 (75%) out of 32 patients, and without significant adverse events[65]. Minor side effects of CPN/B including abdominal pain, diarrhea and hypotension due to autonomic nervous system disruption are usually self-limiting. Rare serious adverse events were reported in case reports, including fatal celiac artery thrombosis causing infarction[66], paralysis from anterior spinal cord infection[67] and necrotic gastric perforation[68]. Given the high efficacy of EUS-guided EUS-CPN/B and rarity of adverse events, the latest (1/2020) version of the National Comprehensive Cancer Network (NCCN) guidelines, recommends EUS-CPN for pain palliation in severe pain unresponsive to around-the-clock analgesics or undesirable analgesics side effects[69] (Table 3).

COMBINED EUS AND ERCP IN PANCREATIC ADENOCARCINOMA TREATMENT

Bile duct obstruction with resultant obstructive jaundice and occasional-disabling pruritus is among the most common symptoms of pancreatic head adenocarcinoma. It is usually drained through bile duct stenting introduced *via* ERCP. Employing the beneficial effect of brachytherapy using the radioactive seeds iodine-125, Liu *et al*[70]

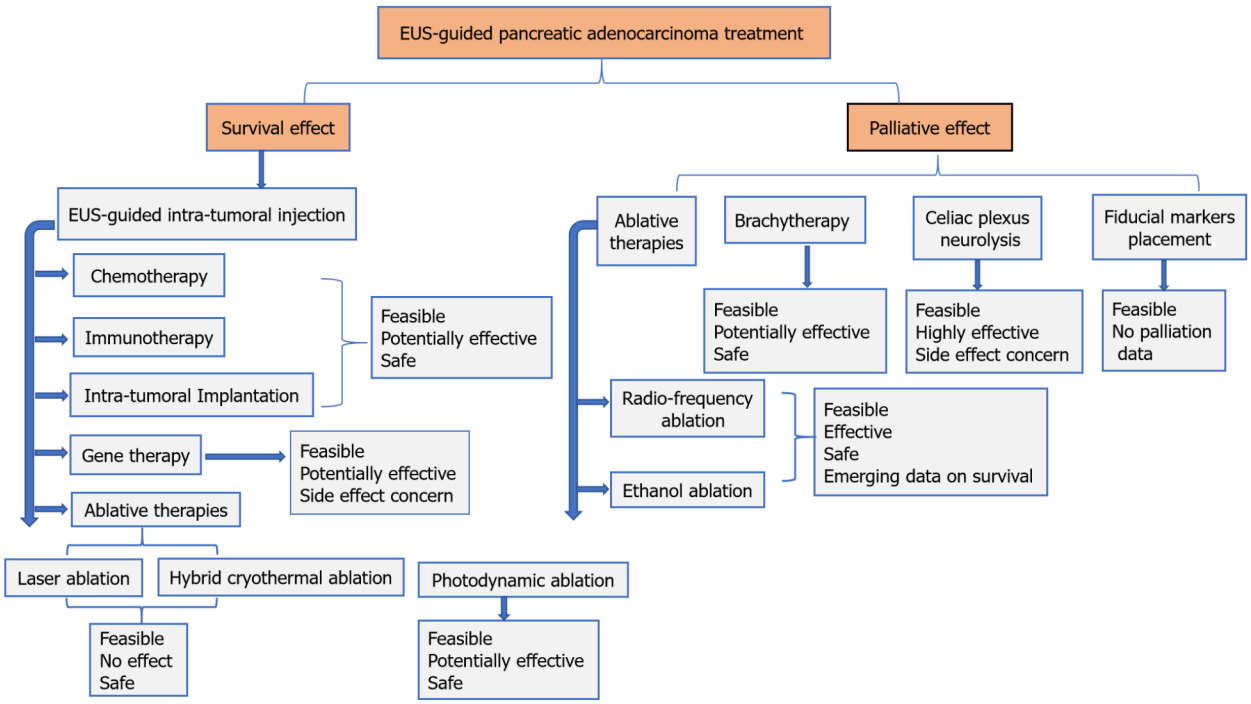

Figure 1 Demonstrates the available endoscopic ultrasound-guided treatment options in pancreatic adenocarcinoma. EUS: Endoscopic ultrasound.

reported that brachytherapy through a preloaded pancreatic stent with iodine-125 seeds, was feasible and safe in an animal experiment using pigs[70]. Two years later, the Liu *et al*[71] group reported the feasibility and tolerability, in a pilot study, of combined radioactive stents with metallic and/or plastic stent in peripancreatic head advanced carcinomas, with stable disease in 72.7% of patients[71]. A recent retrospective study evaluated the role of EUS and/or percutaneous ultrasound-guided iodine-125 seed implantation in 50 patients with unresectable pancreatic carcinoma combined with prior biliary stenting *via* ERCP *vs* biliary stenting alone in 51 patients. They reported longer survival, increased pain reduction with improved life quality, postponed gastric outlet obstruction and longer stent patency in the combination treatment group[72].

SUMMARY

Overall, we identified 12 prospective studies including 261 patients, most in stage III of disease, that utilized EUS-guided intra-tumoral injection therapies and mainly reported effect on patient survival. These studies reported complete technical success without significant effect on overall survival rate, but with several severe adverse events varying in occurrence among the studies. Similarly, in the EUS-guided ablation therapies, we identified one retrospective and eight prospective studies that included 174 patients and reported excellent technical success and minor adverse events, but an inconclusive effect on survival, as half of the studies were feasibility studies not reporting overall survival. Only two of those studies reported pain palliation, however the palliation was significant, thus leading to a hope for performing this treatment for palliative purposes. Finally, we identified seven studies on FMPs, most of them were feasibility studies showing high technical success and minor adverse events. Four studies on brachytherapy included 87 patients and four studies on CPN including 179 patients, with significant improvement in pain ranging from 33% to 90% of patients, no survival benefit and no serious procedure-related adverse events (Table 4).

CONCLUSIONS

In recent years we have witnessed a great advance in interventional and therapeutic EUS. With these developments, EUS has become the preferred alternative for intra-

tumoral injections, ablative therapies, implantation therapies, FMP, brachytherapy, and CPN/B in advanced pancreatic adenocarcinoma. Most EUS-FNI treatments are still far from optimal, and are still in their early stage with little available data, generated from small trials. Figure 1 summarizes the available treatment options for pancreatic adenocarcinoma. The EUS-guided ablation therapies, although encouraging, are far from being standardized. These techniques are in the midst of a long process, necessitating the performance of large prospective randomized controlled studies that compare the different treatment approaches combined with chemo +/- radiotherapy, with respect to success, efficacy, safety and survival. Finally, EUS-guided FMP and brachytherapy are easy, safe and promising modalities, but studies comparing them with the conventional approach of radiotherapy are lacking, while EUS-guided CPN/B is a feasible and accepted tool in pancreatic cancer-related pain control.

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World Journal of Gastroenterology

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World J Gastroenterol 2022 January 21; 28(3): 348-364

DOI: 10.3748/wjg.v28.i3.348

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Basic Study

Melatonin prevents oxidative stress, inflammatory activity, and DNA damage in cirrhotic rats

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Author contributions: Colares JR, Marroni CA and Marroni NP participated in study design and development, data analysis and interpretation, and writing the manuscript; Schemitt EG, Hartmann RM, Fonseca SRB, Brasil MS, Bueno AF and Dias AS, participated in the oxidative stress and immunohistochemistry analyses and interpretation of results.

Institutional review board statement: This study was conducted at the Laboratory of Experimental Hepatology and Gastroenterology, Hospital de Clínicas of Porto Alegre after approval from the Institutional Animal Care and Use Committee (opinion no. CEUA-HCPA No. 2016-0373).

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Abstract

BACKGROUND

Cirrhosis is an important health problem characterized by a significant change in liver parenchyma. In animals, this can be reproduced by an experimental model of bile duct ligation (BDL). Melatonin (MLT) is a physiological hormone synthesized from serotonin that has been studied for its beneficial properties, including its antioxidant potential.

AIM

To evaluate MLT's effects on oxidative stress, the inflammatory process, and DNA damage in an experimental model of secondary biliary cirrhosis.

METHODS

Male Wistar rats were divided into 4 groups: Control (CO), CO + MLT, BDL, and BDL + MLT. MLT was administered (20 mg/kg) daily beginning on day 15 after biliary obstruction. On day 29 the animals were killed. Blood samples, liver tissue, and bone marrow were collected for further analysis.

Institutional animal care and use committee statement: This study was conducted at the Animal Experimentation Unit, Hospital de Clínicas of Porto Alegre after approval from the Institutional Animal Care and Use Committee (opinion no. CEUA-HCPA No. 2016-0373).

Conflict-of-interest statement: The authors declare that they have no conflicts of interest.

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

Supported by Fundo de Incentivo à Pesquisa e Eventos (FIPE).

Country/Territory of origin: Brazil

Specialty type: Gastroenterology and hepatology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Received: May 5, 2021
Peer-review started: May 5, 2021
First decision: June 12, 2021
Revised: June 24, 2021
Accepted: January 11, 2022
Article in press: January 11, 2022
Published online: January 21, 2022

RESULTS

BDL caused changes in biochemical and histological parameters and markers of inflammatory process. Thiobarbituric acid (0.46 ± 0.01) reactive substance levels, superoxide dismutase activity (2.30 ± 0.07) and nitric oxide levels (2.48 ± 0.36) were significantly lower ($P < 0.001$) n the groups that received MLT. DNA damage was also lower ($P < 0.001$) in MLT-treated groups (171.6 ± 32.9) than the BDL-only group (295.5 ± 34.8). Tissue damage and the expression of nuclear factor kappa B, interleukin-1β, Nrf2, NQO1 and Hsp70 were significantly lower in animals treated with MLT ($P < 0.001$).

CONCLUSION

When administered to rats with BDL-induced secondary biliary cirrhosis, MLT effectively restored the evaluated parameters.

Key Words: Antioxidants; Secondary biliary cirrhosis; Oxidative stress; Melatonin; Bile duct ligation

Core Tip: The experimental model of secondary biliary cirrhosis, by ligation of the main bile duct, mimics the clinical situation, with biochemical, enzymatic, histological changes, and similar biological, inflammatory, genotoxic markers and oxidative stress triggers. Melatonin, used as an antioxidant therapeutic agent, has been shown to be effective in reversing the changes caused at different levels due to its antioxidant, anti-inflammatory, cytoprotective action, including a reduction in DNA damage, with significant improvement and future therapeutic potential.

Citation: Colares JR, Hartmann RM, Schemitt EG, Fonseca SRB, Brasil MS, Picada JN, Dias AS, Bueno AF, Marroni CA, Marroni NP. Melatonin prevents oxidative stress, inflammatory activity, and DNA damage in cirrhotic rats. *World J Gastroenterol* 2022; 28(3): 348-364
URL: https://www.wjgnet.com/1007-9327/full/v28/i3/348.htm
DOI: https://dx.doi.org/10.3748/wjg.v28.i3.348

INTRODUCTION

Secondary biliary cirrhosis, a late complication of prolonged obstruction of the extrahepatic bile duct, causes cholestasis[1]. Cholestatic liver damage, defined according to histopathological and biochemical criteria as an accumulation of toxic bile acids, plays a fundamental role in liver necrosis and fibrosis[2-4].

Prolonged common bile duct obstruction in rats is an experimental model that induces secondary biliary cirrhosis in 28 d and mimics human liver disease in clinical, laboratory and histological parameters[2,3,5].

Oxidative stress plays a determinant role in the pathophysiology of liver diseases due to the accumulation of reactive oxygen species, protein oxidation, lipid peroxidation (LPO) and DNA damage, which has also been evaluated in different experimental models[3,5-7].

Oxidative stress, due to the accumulation of reactive oxygen species, destabilizes cell homeostasis. Nrf2, which regulates cellular response to oxidative damage and the expression of most antioxidant enzymes under normal conditions, is kept inactive by the protein Keap1. In stressful situations for the cell, Nrf2 dissociates and translocates to the nucleus, where it binds to the promoter sequence as an antioxidant response element and activates genes, initiating the transcription of new antioxidant enzymes[8-10]. The Keap1/Nrf2 pathway is responsible for regulating both cytoprotective genes and defense antioxidants, including superoxide dismutase (SOD), catalase, and glutathione peroxidase, as well as glutathione reductase, gamma glutamylcysteine ligase, xenobiotic detoxification, NAD (P) H: Quinone oxidoreductase 1 (NQO1), and genes from the glutathione S-transferase family[11,12].

P-Reviewer: Goyal AK, Skrypnik D
S-Editor: Fan JR
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Increased production of reactive oxygen species, the presence of inflammatory mediators such as interleukins (IL-1β and IL-6), tumor necrosis factor alpha (TNF-α), nuclear factor kappa B (NF-kB), and increased nitric oxide levels may be related to the development of fibrosis in liver cirrhosis[2,3].

Hsp70 is an endogenous protein that plays a protective role in cell function, assisting in protein synthesis. Studies have shown that Hsp70 induction occurs in response to various stimuli, such as exposure to toxins, glucose deprivation, and reactive oxygen species formation, as well as liver cirrhosis[13,14].

Melatonin (MLT), N-acetyl-5-methoxytryptamine, is a hormone synthesized by the pineal gland, which is produced rhythmically and is inhibited by light[15,16]. The antioxidant effect of MLT is related to its amphiphilic chemical structure, which facilitates its crossing through biological barriers and allows activity in both aqueous and lipid environments[15,17]. The numerous attributes of MLT include antioxidant capacity and anti-inflammatory and immunomodulatory effects[18]. Exogenous MLT has protective effects on hepatic ischemia-reperfusion injury[19]. The inadequate expression of MLT predisposes liver cells to immune- and oxidative stress-related damage. MLT, *via* epigenetic modulation, was able to suppress NF-κB signaling activation and protecting against apoptotic signaling induced by either oxidative stress or high concentrations of bile[20]. MLT, participates in regulating multiple physiological functions, including sleep, circadian rhythms, and neuroendocrine processes.

Current evidence shows that MT protects against liver injury by inhibiting oxidation, inflammation, haematopoietic stem cell (HSC) proliferation, and hepatocyte apoptosis, thereby inhibiting the progression of liver cirrhosis[17].

Inflammation and oxidative stress play an important role in the pathophysiology of cirrhosis and other liver diseases, which is why pharmacological interventions can change the evolution of the disease. Non-alcoholic fatty liver disease (NAFLD) patients who underwent treatment with Essentiale Forte and tryptophan or MT for 14 mo had reduced expression of GGTP, triglycerides, low-density lipoprotein cholesterol and proinflammatory cytokines including IL-1, IL-6 and TNF-α, although there was no significant difference in alanine aminotransferase level or other biochemical parameters[21]. NAFLD patients treated with MT were found to have significantly lower aspartate aminotransferase (AST) and high-sensitivity C-reactive protein levels and a better liver grade than those who received placebo[22]. MLT seems safe and effective in the short term as a sedative in patients with CTP classes A and B cirrhosis and SD. This finding may have clinical applications in the holistic management of patients with cirrhosis[23]. There is a positive association between high serum MLT levels prior to LT, one-year survival after LT, and total antioxidant capacity[24].

The aim of the present study was to evaluate MLT's effects on oxidative stress, the inflammatory process, and DNA damage in an experimental model of secondary biliary cirrhosis.

MATERIALS AND METHODS

Ethical considerations

This study was conducted at the Animal Experimentation Unit and the Second Laboratory of Experimental and Inflammatory Pneumological Sciences of the Hospital de Clínicas de Porto Alegre after approval by the Institutional Commission for the Treatment and Use of Animals (protocol 2016-0373).

Animal handling was carried out according to Brazilian federal legislation (Law 11794/2008), Brazilian Council for the Control of Animal Experimentation (CONCEA) rules, the State Code for the Protection of Animals, and local legislation regarding the care and use of animals in experimental research.

Experimental procedures

Twenty-four male Wistar rats (mean weight 300 g) were divided into four experimental groups: Control (CO), control treated with MLT (CO + MLT), bile duct ligation (BDL) and BDL treated with MLT (BDL + MLT). During the experiment, the animals were maintained in cages (47 cm × 34 cm × 18 cm) lined with wood shavings, under a 12 h light/dark cycle and controlled temperature (18-22 °C), with free access to food and water.

On the first day of the experiment, BDL surgery was performed, as well as simulated surgery in the CO and CO + MLT groups according to Kountouras *et al* (1984)[25]. On the 15th day of the experiment, the animals began receiving MLT in daily

doses of 20 mg/kg of body weight. The treatment continued until the 28th day.

On the 29th day, the animals were weighed and anesthetized by intraperitoneal injection of a mixture of ketamine hydrochloride (95 mg/kg) and 2% xylazine hydrochloride (8 mg/kg). Blood was then collected from the retro-orbital plexus with a glass capillary tube and placed in a test tube with heparin to prevent coagulation.

After blood collection, the animals were sacrificed by anesthetic overdose (three times the therapeutic dose, according to the CONCEA guidelines. Upon confirmation of death, a ventral midline laparotomy was performed, after abdominal trichotomy and disinfection. The liver was removed, sectioned, and stored for subsequent analysis. One liver fragment was submerged in a 10% formaldehyde solution for 24 h for histological examination, one fragment was stored in a fixative containing glutaraldehyde for subsequent analysis by scanning electron microscopy, a third fragment was frozen at -80 °C for further analysis, and bone marrow samples were collected for the micronucleus test.

Histological analysis of hepatic tissue

After dissection, the liver was placed in 10% buffered formalin and later embedded in paraffin blocks. The paraffin blocks were then attached to a microtome (Leitz-1512 Microtome, Leitz, Wetzlar, Germany) for cutting. The slides were then stained with hematoxylin-eosin and washed in running water. In the dehydration phase, the structures went through a series of three baths: One in absolute alcohol and two in xylol. The cover slip was then fixed into place using Canada balsam or Entellan, which completed the preparation process. The slides were analyzed with a Nikon Labophot binocular microscope equipped with a digital camera. Using the Image-Plus software (Media Cybernetics, Bethesda, MD, United States), images were captured at different magnifications.

Microscopy evaluation

Tissue samples were fixed in 10% formalin and embedded in paraffin. The paraffin blocks were then attached to a microtome (Leitz® 1512) and cut in 3 μm sections. The slides were stained in hematoxylin-eosin for 5 min each and then washed in running water. In the dehydration phase, the structures went through a series of three baths: One in absolute alcohol and 2 in xylol. The cover slip was fixed into place using Canada Balsam. The slides were analyzed with a microscope equipped with a digital camera. Using Image-Plus software, images were captured at 200× magnification.

Preparation of homogenates

Nine ml of phosphate buffer were added *per* gram of tissue, which was then homogenized in an Ultra-Turrax homogenizer (IKA-Werk, Staufen, Germany) for approximately 40 s and kept on ice, followed by centrifugation in a SORVALL RC-5B refrigerated Superspeed Centrifuge (Du Pont Instruments, Miami, FL, United States) for 10 min at 4000 rpm[26]. The precipitate was discarded and the supernatant was used to quantify the proteins.

Microscopic analysis of liver tissue

Liver samples were collected for scanning electron microscopy. After collection, the samples were immersed in a fixative solution containing glutaraldehyde. The samples were then washed, dehydrated, desiccated and metallized, followed by analysis in an electron microscope (Jeol JSM-T330, Tokyo, Japan) at 5000× magnification.

Analysis of biochemical and spectrophotometric parameters

Liver integrity was assessed by measuring liver enzymes AST, alanine aminotransferase, and alkaline phosphatase in plasma with a Liquiform Labtest® kit (a kinetic spectrophotometric assay).

The protein content in liver homogenate was determined using the Bradford method[27]. LPO was investigated with a thiobarbituric acid reactive substances (TBARS) assay, with the concentration expressed in nmol/mg of protein[28]. SOD activity was measured in a plate reader, evaluating its ability to inhibit the superoxide radical from reacting with adrenaline. The results were expressed in SOD units *per* milligram of protein[29]. The production of nitric oxide metabolites [nitrites (NO_2) /nitrates(NO_3)] was measured indirectly with the Griess reaction. This assay is based on the enzymatic reduction of NO_3 to NO_2 in the presence of nitrate reductase and NQO1, with subsequent colorimetric determination of NO_2 using the Griess reagent (a mixture of sulfanilamide and NO_2-specific *N*-[1-Naphthyl]ethylenediamine). The results were expressed in mmol/L[30].

DNA damage analysis

To assess DNA damage, we used the alkaline version of the comet assay described by Tice *et al* (2000)[31]. Aliquots of 10 ml of liver cell suspension were mixed with 0.75% low-melting agarose and placed 1.5% agarose-coated slides; these slides were immersed in a lysis solution, which allowed the migration of DNA fragments by electrophoresis. The results were expressed in a damage index, obtained by visual assessment of damage classes (from 0 to 4), and damage frequency, calculated from the number of cells with *vs* without tails[32].

As described by Mavournin *et al* (1990)[33], bone marrow samples were collected from both femurs for the micronucleus test. To collect the samples, the proximal end of each femur was cut to expose the spinal canal, allowing extraction. To count normochromatic erythrocytes, polychromatic erythrocytes (PCE), and micronuclei in the PCE, an optical microscope with an immersion objective was used, and at least 2000 PCE were analyzed *per* animal. The polychromatic/normochromatic erythrocyte ratio was also determined by assessing the frequency of PCE in 1000 erythrocytes from each animal[34].

Multiplex analysis

IL-1β cytokine levels were assessed using a microsphere-based multiplex assay (MILLIPLEX Map Kit, Rat Cytokine/Chemokine Magnetic Bead Panel, Cat. Nº. RECYTMAG-65K; Millipore Corporation, Billerica, MA, United States). Cytokine detection was performed by adding specific fluorescence conjugated antibodies.

Quantification was based on a standard curve with known dilutions, and the results were expressed in pg/mg. The samples were analyzed in a Luminex 200TM reader (Luminex, Austin, TX, United States) according to manufacturer instructions.

Immunohistochemical analysis

The slides, pre-incubated with 10% rabbit serum at room temperature to block possible unwanted reactions from the secondary antibody, were incubated with monoclonal antibodies (Nrf2, NQO1, NF-κB and Hsp70) (Santa Cruz Biotechnology, Santa Cruz, CA, United States) overnight at 4 ºC, followed by incubation with a secondary antibody for one hour at room temperature. After 60 min at room temperature, they were treated with EnVision reagent and washed three times with phosphate-buffered saline. The nuclei were counterstained with hematoxylin. The primary antibody was diluted in phosphate-buffered saline, which contained bovine albumin as a negative control. The results were evaluated by blinded pathologists using a microscope equipped with a digital camera and Image-Plus software (Media Cybernetics).

Western blot analysis

Cytoplasmic and nuclear extracts were prepared from liver homogenates using a specific lysis buffer and protease inhibitors[35]. The supernatant fraction was collected and stored in aliquots at -80 °C for further analysis. The lysed proteins were separated by dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were then blocked with 5% skim milk in Tris buffer containing 0.05% Tween 20 (TTBS) for 60 min at 37 °C. Thereafter, the primary antibodies were incubated and stirred overnight at 4 ºC. The following proteins were evaluated: NF-kB (65 kDa and Hsp70; 70 kDa) (Santa Cruz Biotechnology, Santa Cruz, CA, United States) diluted from 1:200 to 1:1000 with Tris-buffered saline in skim milk at 5%. HRP-antibody protein biomarker detection was performed with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom). The density of specific bands was quantified through image densitometry software (Scion Image, Frederick, MD, United States)[36,37].

Statistical analysis

The quantitative data were expressed as mean ± standard error. The groups were compared using unilateral analysis of variance. The Student-Newman-Keuls procedure was used to find differences in means (SPSS, version 17.0). The Tukey test was used for the comet assay. The data were analyzed in GraphPad InsTat 3.1, with $P < 0.05$ considered significant.

RESULTS

Histological analysis

In the histological analysis, hematoxylin-eosin staining in the BDL group revealed changes in the liver parenchyma, a loss of hepatocyte cords, and the presence of inflammatory infiltrate (Figure 1, black arrows). In the BDL + MLT group, we observed restructuring of these changes, including the formation of hepatocyte cords, decreased inflammatory infiltrate, and preserved hepatocytes (Figure 1). In the CO and CO + MLT groups, the liver parenchyma was unchanged.

Scanning electron microscopy analysis

According to scanning electron microscopy of the liver samples, the ciliated membrane, which covers hepatocytes involved in inflammatory process signaling in response to damage, was intact in the CO and the CO + MLT groups. This membrane was damaged in the BDL group, although in the BDL + MLT group the membrane had been restructured (Figure 2).

Analysis of biochemical and spectrophotometric parameters

Liver enzyme alterations: All plasma liver enzymes in the BDL group were significantly higher than in the control groups (CO and CO + MLT), and these values were significantly lower in the BDL + MLT group than the BDL group ($P < 0.001$) (Figure 3).

Protein evaluation, LPO, antioxidant enzyme SOD and nitric oxide: The total protein levels (Figure 4A) in liver homogenate were significantly higher in the BDL group than the control groups ($P < 0.001$) and were significantly higher in the BDL + MLT group than the BDL group ($P < 0.001$).

The LPO level was significantly higher in the BDL group than the CO and CO + MLT groups and was significantly lower in the BDL + MLT group than the BDL group ($P < 0.001$) (Table 1).

There was significantly less SOD activity in the BDL group than the control groups (CO and CO + MLT) and significantly more activity in the BDL + MLT group than the BDL group ($P < 0.001$) (Table 1).

The levels of nitric oxide metabolites (nitrites and nitrates) were significantly higher in the BDL group than the CO and CO + MLT groups, but they were significantly lower in the BDL + MLT group than the BDL group ($P < 0.001$) (Table 1).

DNA damage analysis

In the comet assay analysis, the BDL group had a significantly higher damage index and damage frequency than the CO and CO + MLT groups. These parameters were significantly lower in the BDL + MLT group than the BDL group ($P < 0.001$) (Table 2).

The micronucleus frequency was significantly higher in the BDL group than the CO and CO + MLT groups and was significantly lower in the BDL + MLT group than the BDL group ($P < 0.001$) (Table 3). No significant differences were found between the groups in the polychromatic/normochromatic erythrocyte ratio, which indicated no toxicity in the bone marrow.

Multiplex analysis

The pro-inflammatory cytokine IL-1β levels were significantly higher in the BDL group than the CO and CO + MLT groups ($P < 0.001$) and were significantly lower in the BDL + MLT group than the BDL group ($P < 0.001$), as can be seen in Figure 4B.

Immunohistochemistry and quantification of Nrf2, NF-kB, Hsp70 and NQO1

We observed significantly higher expression of NF-kB (Figure 5A) and Hsp70 (Figure 6A) in the BDL group than the control groups and significantly lower expression in the BDL + MLT group than the BDL group ($P < 0.001$).

There was significantly lower expression of Nrf2 in the BDL group than in the CO and CO + MLT groups but significantly higher expression in the BDL + MLT group than the BDL group (Figure 7). There was significantly higher expression of NQO1 in the BDL group than the CO and CO + MLT groups but significantly lower expression in the BDL + MLT group than the BDL group ($P < 0.001$)(Figure 8).

Expression of NF-kB and Hsp70

Western blot analysis of NF-kB (Figure 5B) and Hsp70 (Figure 6B) expression showed that they were significantly higher in the BDL group than the control groups (CO and

Table 1 Activity of lipid peroxidation levels, antioxidant enzyme superoxide dismutase, and nitric oxide levels in the experimental groups

Groups	TBARS	SOD	NO_2/NO_3
CO	0.29 ± 0.01	2.62 ± 0.11	1.69 ± 0.08
CO + MLT	0.34 ± 0.02	2.43 ± 0.05	1.76 ± 0.09
BDL	3.43 ± 0.08[a]	0.61 ± 0.05[a]	5.53 ± 0.30[a]
BDL + MLT	0.46 ± 0.01[b]	2.30 ± 0.07[b]	2.48 ± 0.36[b]

[a]$P < 0.001$: Significant increase in the bile duct ligation group in relation to the control and CO + melatonin groups.
[b]$P < 0.001$: Significant reduction in the BDL + MLT group in relation to the BDL group.
CO: Control; MLT: Melatonin; BDL: Bile duct ligation; TBARS: Thiobarbituric acid reactive substances; SOD: Superoxide dismutase.

Table 2 Comet assay in liver tissue of rats that underwent bile duct ligation treated or not with melatonin

Group	Damage index	Damage frequency
CO	77.4 ± 8.6	72.2 ± 7.3
CO + MLT	72.6 ± 16.2	67.8 ± 14.8
BDL	295.5 ± 34.8[a]	100.0 ± 0.0[c]
BDL + MLT	171.6 ± 32.9[b]	91.6 ± 9.5

The damage index can range from 0 (completely undamaged, 100 cells × 0) to 400 (with maximum damage 100 × 4). The damage frequency was calculated based on the number of cells with *vs* without tails.
[a]$P < 0.001$: Significant increase in the bile duct ligation group in relation to the control and CO + melatonin groups.
[b]$P < 0.001$: Significant reduction in the BDL + MLT group in relation to the BDL group.
[c]$P < 0.01$: Significant increase in the BDL group in relation to the CO and CO + MLT groups.
CO: Control; MLT: Melatonin; BDL: Bile duct ligation; TBARS: Thiobarbituric acid reactive substances.

Table 3 Micronucleus test in the bone marrow of the rats that underwent bile duct ligation, treated or not with melatonin

Groups	MNPCE[1] in 2000 PCE	Ratio PCE/NCE[2]
CO	4.0 ± 1.5	1.1 ± 0.2
CO + MLT	3.3 ± 1.0	1.1 ± 0.4
BDL	7.8 ± 1.3[a]	1.0 ± 0.6
BDL + MLT	6.8 ± 1.9	0.9 ± 0.5

[1]MNPCE: Micronuclei in polychromatic erythrocytes.
[2]PCE/NCE ratio: Proportion of polychromatic erythrocytes/normochromatic erythrocytes.
[a]$P < 0.01$: Significant increase in the bile duct ligation group in relation to the control and CO + melatonin groups. (Analysis of variance, Tukey test).
CO: Control; MLT: Melatonin; BDL: Bile duct ligation.

CO + MLT) and significantly lower in the BDL + MLT group than the BDL group ($P < 0.001$).

DISCUSSION

Common bile duct obstruction causes hepatocellular damage and an inflammatory response due to the accumulation of bile salts in the liver, which promotes cytokine production, hepatocellular injury, and the healing process, leading to an accumulation of collagen, fibrosis, and liver cirrhosis[38]. The BDL model has been used to study the numerous molecular signaling pathways involved in secondary biliary cirrhosis[3].

MLT has shown protective effects in different models, inhibiting oxidative stress, inflammatory signaling, autophagy, hepatocyte apoptosis, cell and tissue damage[3,16,

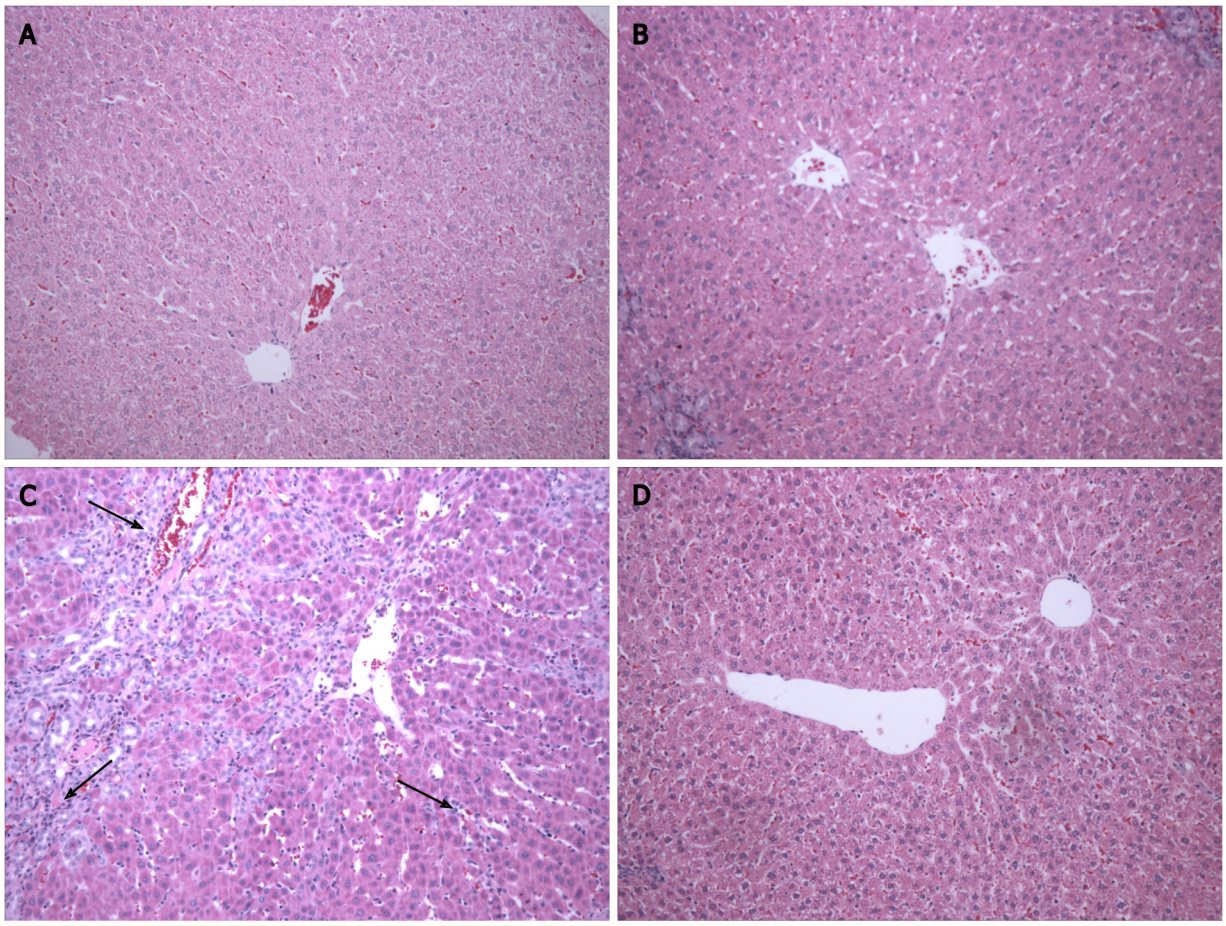

Figure 1 Photomicrograph of hepatic tissue at 200 × magnification in the different experimental groups. The control (CO) and CO + melatonin (MLT) groups had normal liver parenchyma. There was inflammatory infiltrate (black arrows) and a change in the parenchyma in the bile duct ligation (BDL) group. Parenchymal restructuring occurred in the BDL + MLT group. A: CO; B: CO + MLT; C: BDL; D: BDL + MLT.

17,39-41], and it seems safe and effective in the short term as a sedative in patients with CTP classes A and B cirrhosis and SD. This finding may have clinical applications in the holistic management of patients with cirrhosis[23].

The present study investigated the effects of MLT on oxidative, inflammatory, tissue, and cellular injury in an experimental model of secondary biliary cirrhosis. It was found that a dose of 20 mg/kg of MLT, which has already been investigated in other studies by our group, was effective in reducing or modulating oxidative stress, inflammatory processes, and DNA damage. The animals that underwent BDL surgery had a higher expression of the enzymes AST, alanine aminotransferase and alkaline phosphatase than the other groups, indicating possible damage to hepatocyte membranes. When MLT was administered to these animals, we observed a significant decrease in the expression of these enzymes, possibly due to hepatocyte membrane restructuring and reduced liver damage. This corroborates the results of Wu *et al*[12] in a model of BDL and CCL4-induced hepatic fibrosis. These authors found a significant increase in AST and alanine aminotransferase expression, as well as an equally significant reduction in expression after treatment with the antioxidant quercetin. In a model of severe acute liver failure, Schemitt *et al*[42] found an increase in AST, alanine aminotransferase and alkaline phosphatase expression, indicating a loss of liver integrity; high serum levels of these enzymes were related to cell damage and liver cell necrosis, which was reversed with glutamine[42].

Changes in the hepatic parenchyma, including the formation of fibrotic septa and necrosis, are often associated with the process of cirrhosis[12]. Hematoxylin-eosin staining showed disorganized liver tissue in the BDL group, including a loss of hepatocyte cords and the presence of inflammatory infiltrate and fibrosis. However, tissue restructuring had occurred in the BDL + MLT group, and organized tissue was observed in the CO and CO + MLT groups. Scanning electron microscopy revealed that in the control groups (CO and CO + MLT) the ciliated membrane of hepatocytes involved in inflammatory process signaling was intact. In the BDL group, however,

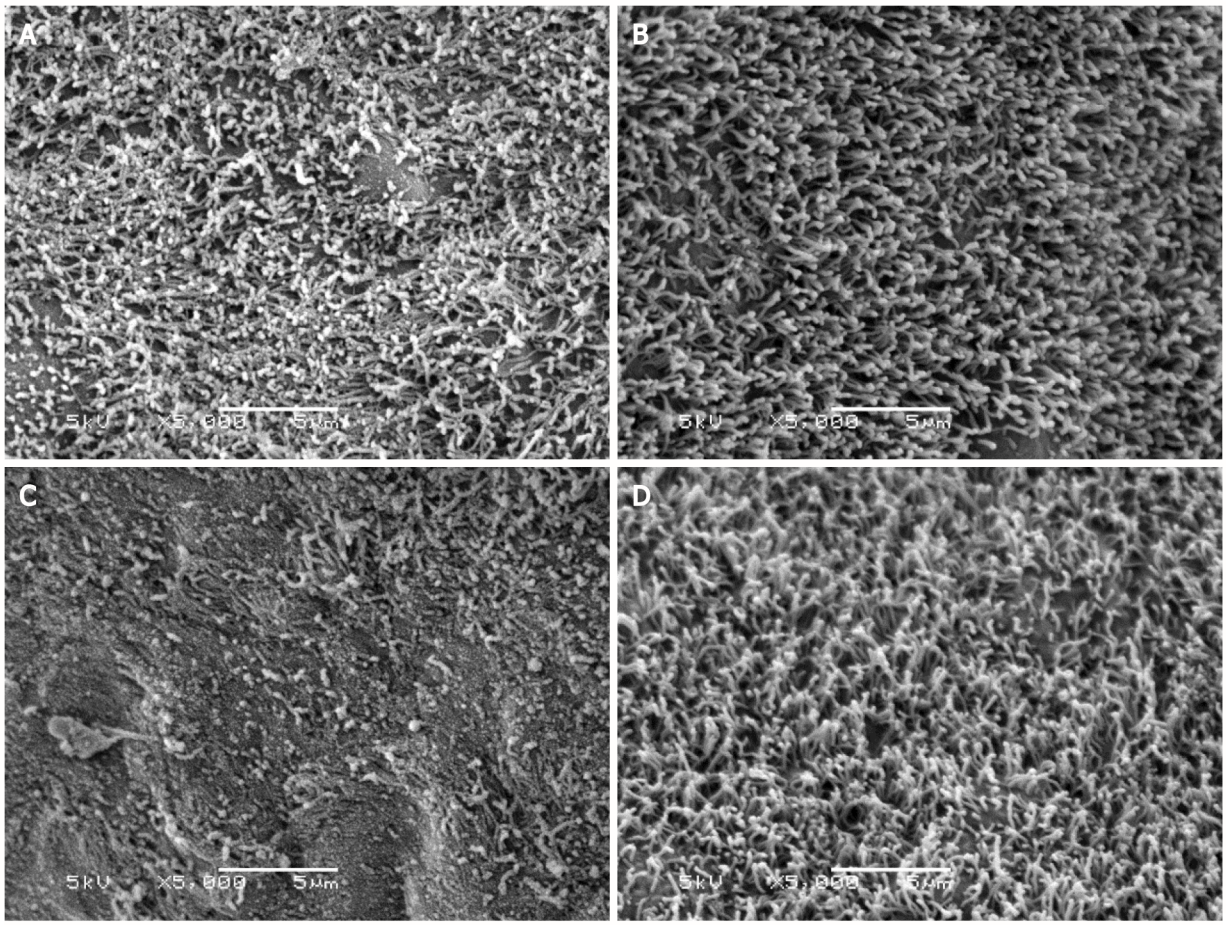

Figure 2 Morphological analysis by scanning electron microscopy of the liver of animals that underwent bile duct ligation surgery. The control (CO) and CO + melatonin (MLT) groups showed an intact ciliated membrane covering the hepatocytes. This membrane is impaired in the bile duct ligation (BDL) group. In contrast, membrane restructuring was observed in the BDL + MLT group. A: CO; B: CO + MLT; C: BDL; D: BDL + MLT.

this membrane was damaged, and in the BDL + MLT group it had been restructured (Figure 2).

In 2016, Wree and Marra[43] described the surface of hepatocytes and their association with reduced cell permeability, as well as a consequent increase in mediators involved in the fibrotic and inflammatory process, which was associated with changes in the ciliated membrane and inflammasome. According to Gonzalez-Navajas[44], inflammation is involved in the pathogenesis of many liver diseases, including cirrhosis, in which many inflammatory cytokines are produced after activation of a multiprotein complex known as inflammasome. However, the origin and mechanisms of hepatic damage mediated by inflammasome are little known[43]. Most acute or chronic liver diseases are accompanied by inflammation, a complex process in response to liver aggression, which causes serious damage to the liver parenchyma[45-47].

In 2017, Giusto *et al*[48] showed that mice with cirrhosis induced by BDL and CCL4 had fibrotic nodules and cellular changes. In 2013, Mazo *et al*[4] observed that in rats submitted in experimental nonalcoholic steatohepatitis, including fibrosis, the administration of N-acetylcysteine restored liver parenchyma.

Studies report that in the pathophysiology of biliary cirrhosis, liver damage is maximized by the action of free radicals. LPO causes the disorganization of cell membranes, resulting in increased membrane permeability and consequent enzyme leakage, leading to cell death. Studies show that plasma malondialdehyde levels may be associated with increased LPO[3,5,42].

In our study, LPO was analyzed with a TBARS assay, and it was significantly higher in the cirrhotic group (BDL) than the other groups, which may be associated with damage to cell membranes. LPO was significantly lower in the BDL + MLT group than the BDL group, which suggests that MLT plays a protective role. These data corroborate a study by Zhu *et al*[49], who, in rats with hepatopulmonary syndrome-BDL induced, observed higher LPO in the cirrhotic group and that Tea polyphenols

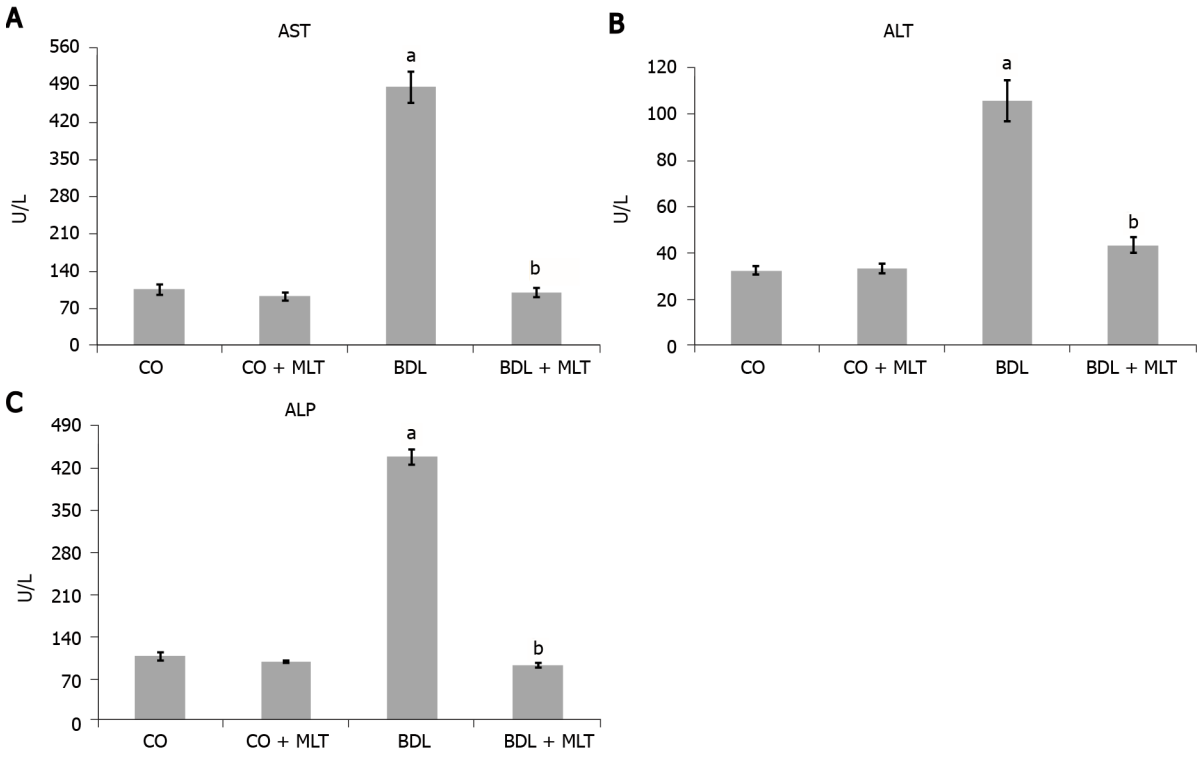

Figure 3 The effect of melatonin on the activity of liver enzymes aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase (alkaline phosphatase) in the plasma of animals that underwent bile duct ligation surgery. The data are expressed as the mean ± standard error of the mean. Significant increase in relation to the control (CO) and the CO + melatonin (MLT) groups ($^aP < 0.001$). Significant decrease in the bile duct ligation (BDL) group in relation to the BDL + MLT group ($^bP < 0.001$). A: AST; B: ALT; C: ALP.

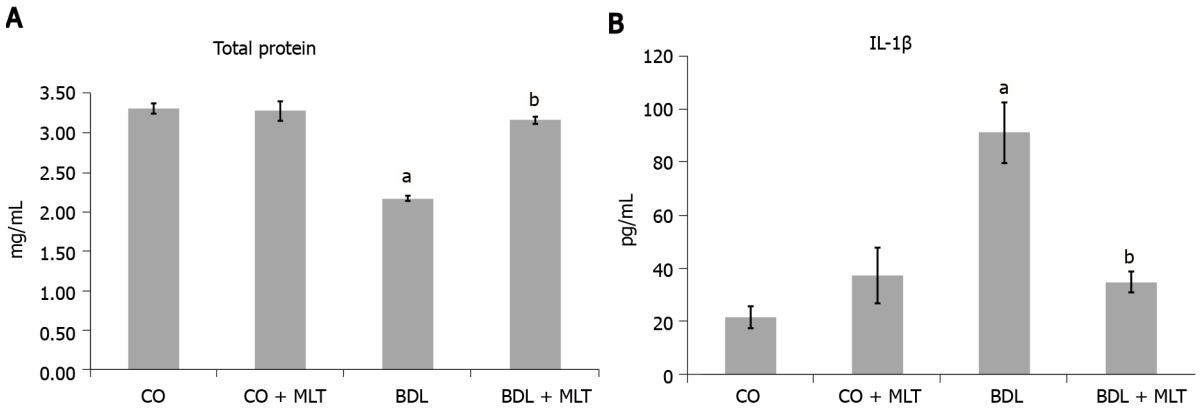

Figure 4 The effect of melatonin on total protein levels and interleukin IL-1β levels in the liver of animals that underwent bile duct ligation surgery. The data are expressed as the mean ± standard error of the mean. Significant decrease/increase in the bile duct ligation (BDL) group in relation to the control (CO) and the CO + melatonin (MLT) groups ($^aP < 0.001$). Significant increase in the BDL + MLT group in relation to the BDL group ($^bP < 0.001$). A: Total protein; B: IL-1β.

significantly decreased LPO. Similar data were observed in other experimental models that administered MLT[3,41,42].

Other authors have observed LPO in the lungs of animals with secondary biliary cirrhosis due to BDL, considering it characteristic of the damage to cell membranes in this experimental model[3,49].

In addition to the lipid damage, which is assessed by increased LPO, reactive oxygen species can damage DNA. Oxidative damage to DNA is common, being the main cause of genomic instability[31]. We observed a significant increase in liver DNA damage in the BDL group, as well as in the frequency of micronuclei in the bone marrow, which suggests increased genomic instability. The significantly better micronucleus and comet assay results in the MLT-treated group suggests that MLT prevented cytogenetic damage, as well as strand breakage and DNA base oxidation in

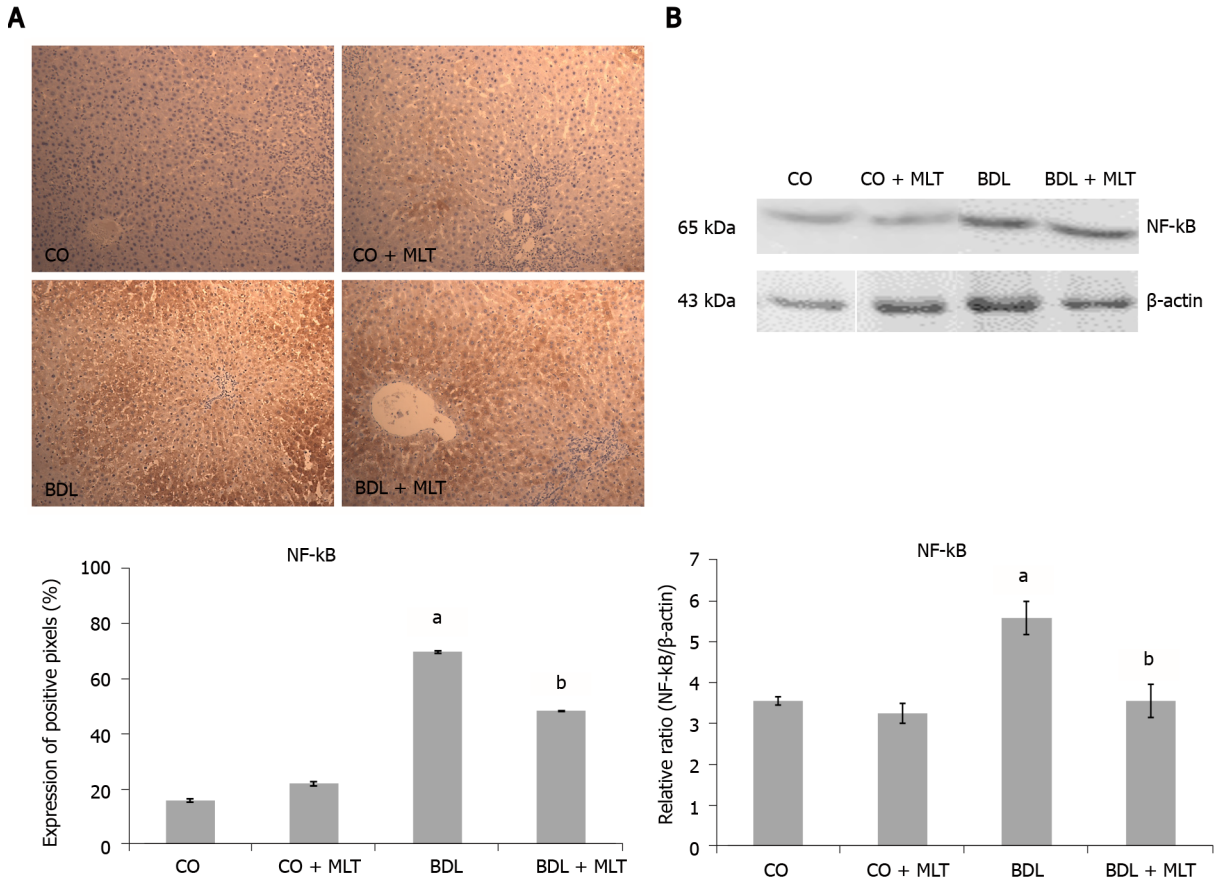

Figure 5 The effect of melatonin on the quantification of immunohistochemistry analysis and the expression of NF-kB with the Western blot technique in the livers of animals that underwent bile duct ligation surgery. The data are expressed as mean ± standard error of the mean. Significant increase in the bile duct ligation (BDL) group in relation to the control (CO) and CO + melatonin (MLT) groups ($^{a}P < 0.001$). Significant decrease in the BDL + MLT group in relation to the BDL group ($^{b}P < 0.001$); A: Expression of positive pixels; B: Relative ratio (NF-kB/β-actin).

this model. The results of the present study corroborate those of Moreira *et al*[40], who used an experimental model of diethylnitrosamine-induced hepatocellular carcinoma, finding a lower damage index and frequency in the MLT-treated group, possibly due to MLT's antioxidant capacity to regulate several key genes involved in DNA repair pathways, in addition to lower oxidative damage, inflammatory processes and tissue damage, as observed in our study. The pathophysiological mechanisms of cirrhosis initiate several signaling pathways, such as the Nrf2 pathway, which has a protective effect against oxidative damage. Under stress conditions, Nrf2 is translocated to the nucleus and activates the expression of genes that encode various antioxidant enzymes, such as SOD and NQO1[17,42,50]. Nuclear expression of Nrf2 was significantly lower in the BDL group in our study. Likewise, we observed that MLT treatment significantly increased Nrf2 expression and reestablished the expression of NQO1 and SOD, which suggests that MLT restored the antioxidant system, reducing oxidative stress by modulating the Nrf2 pathway. These results were similar to those of Schemitt *et al* 2019[42], who evaluated the effects of glutamine in a severe acute liver failure model and observed increased expression of NQO1 and SOD through regulation of the Nrf2-mediated antioxidant system.

In the present study, increased pro-inflammatory cytokine IL-1β levels were observed in the BDL group, as well as increased expression of NF-kB. MLT reversed the inflammatory process, which was demonstrated by lower IL-1β levels and reduced NF-kB expression. Colares *et al*[3] found increased expression of TNF-α and inducible nitric oxide synthase in their BDL group. Inducible nitric oxide synthase is expressed in inflammatory conditions in response to pro-inflammatory cytokines and is associated with increased nitric oxide levels, which we evaluated in the present study. Our results suggest that, due to its anti-inflammatory effect, MLT modulated the NF-kB pathway during inflammation, thus reducing the expression of genes involved in the inflammatory process, such as inducible nitric oxide synthase, nitric oxide and pro-inflammatory cytokines (IL-1β and TNF-α).

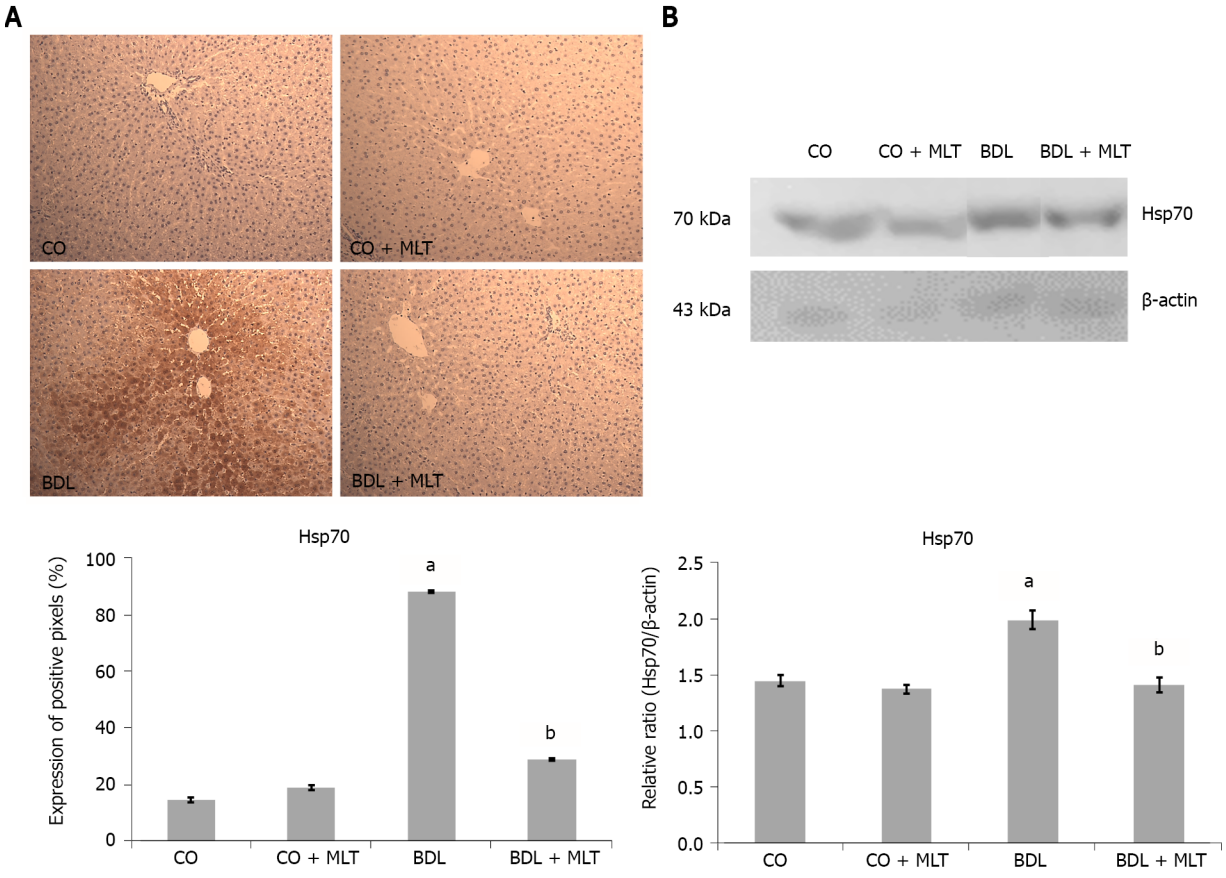

Figure 6 The effect of melatonin on immunohistochemistry and Hsp70 expression with the Western blot technique in the liver of animals that underwent bile duct ligation surgery. The data are expressed as the mean ± standard error of the mean. Significant increase in the bile duct ligation (BDL) group in relation to the control (CO) and CO + melatonin (MLT) groups ($^aP < 0.001$). Significant decrease in the BDL + MLT group in relation to the BDL group ($^bP < 0.001$). A: The effect of melatonin on immunohistochemistry; B: Hsp70 expression with the Western blot technique.

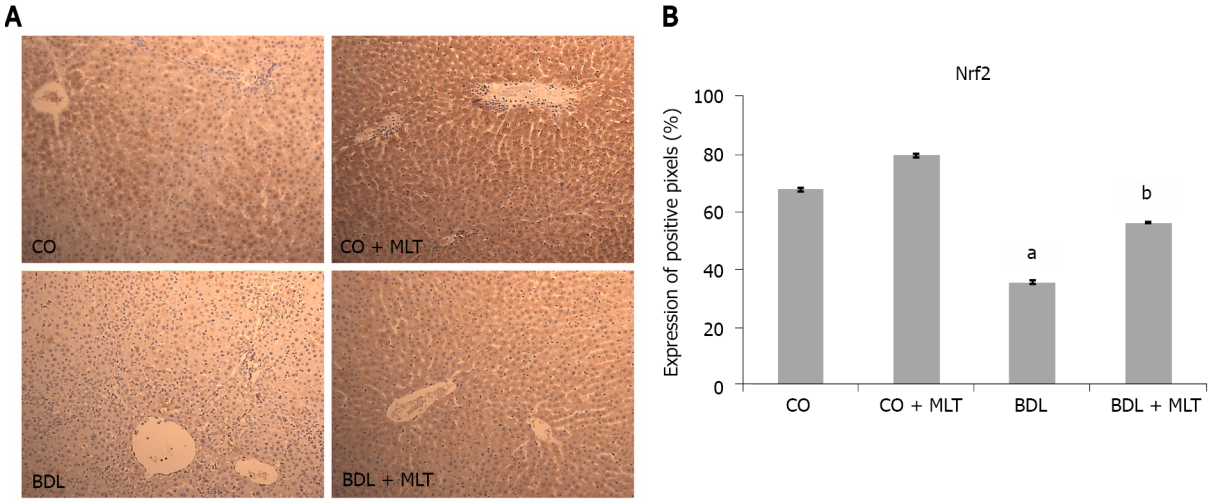

Figure 7 The effect of melatonin on Nrf2 immunohistochemistry in the liver of animals that underwent bile duct ligation surgery. The data are expressed as the mean ± standard error of the mean. A: Express the situation; B: Significant decrease in the bile duct ligation (BDL) group in relation to the control (CO) and CO + melatonin (MLT) groups ($^aP < 0.001$). Significant increase in the BDL + MLT group in relation to the BDL group ($^bP < 0.001$).

In a liver fibrosis model in rats, Czechowska *et al*[51] demonstrated that MLT inhibited the release of NF-kB and, consequently, reduced production of pro-inflammatory cytokines, probably due to its anti-inflammatory antioxidant action. In CCL4-induced liver cirrhosis model in rats, Hardeland[18] observed that MLT reduced the expression of NF-kB, as well as the expression of inducible nitric oxide synthase.

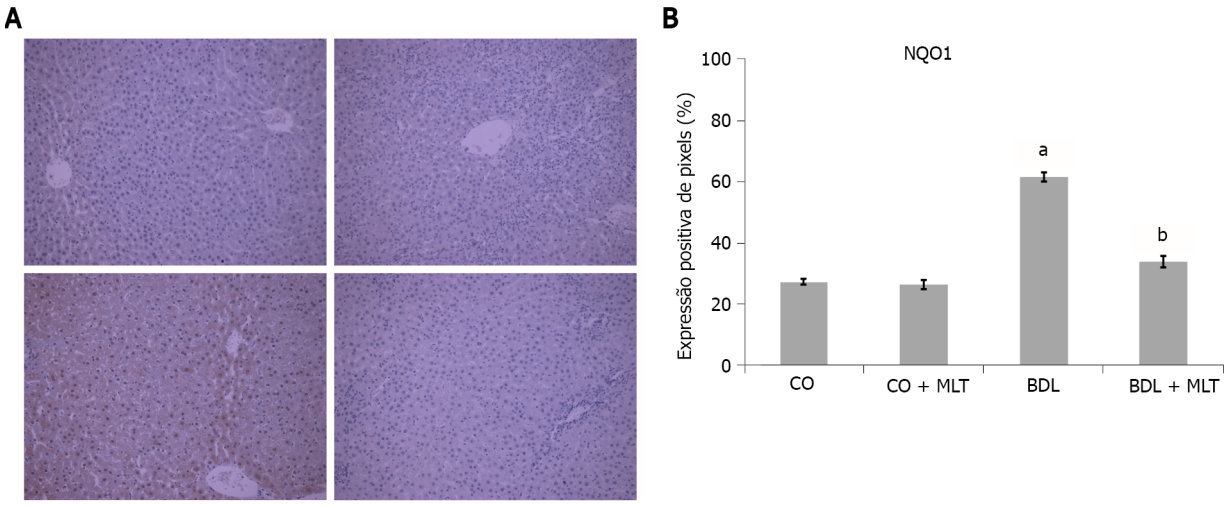

Figure 8 The effect of melatonin on quantification of NQO1 immunohistochemistry in the liver of animals that underwent bile duct ligation surgery. The data are expressed as mean ± standard error of the mean. A: Express the situation; B: Significant increase in the bile duct ligation (BDL) group in relation to the control (CO) and CO + melatonin (MLT) groups ($^{a}P < 0.001$). Significant decrease in the BDL + MLT group in relation to the BDL group ($^{b}P < 0.001$).

Heat shock proteins are extremely important for the protection of cells. In particular, Hsp70, which has cytoprotective functions, acts on protein folding, transport and degradation and can be induced in response to various stresses, including trauma, inflammatory diseases, oxidative stress and liver cirrhosis[52]. We observed an increase in the expression of Hsp70 in the animals of the BDL group, however, treatment with MLT led to a significant reduction in the expression of Hsp70. This suggests that MLT, possibly due to its important antioxidant effect, effectively regulated Hsp70 in the BDL + MLT group. In 2015, Moreira *et al*[40] reported an increase in Hsp70 expression in a model of hepatocellular carcinoma in rats treated with MLT. In 2019, Schemitt *et al*[42] evaluated a model of liver toxicity and observed that the expression of Hsp70 was reduced, possibly due to the increase in oxidative stress, thus contributing to disease worsening.

Biosynthesis of MLT by cholangiocytes is essential for maintaining biliary epithelium function and this cytoprotective mechanism, which appears to be impaired by decreased biliary MLT synthesis in biliary duct obstruction, exacerbates biliary damage and liver fibrosis. Concomitant with enhanced liver fibrosis, we observed increased biliary senescence[20].

MLT has been demonstrated to ameliorate liver damage by decreasing oxidative stress, inflammatory responses, and bile acid-induced apoptosis. The inadequate expression of MLT predisposes liver cells to immune- and oxidative stress-related damage[53].

In cholangiocytes exposed to mitochondrial oxidative stress, MLT decreased the expression of proapoptotic stimuli, which was accompanied by the inhibition of NFκB-p65, a pivotal mediator of inflammatory response, activation of antiapoptotic signaling, and increased biliary senescence and ROS, which activated HSCs by a paracrine mechanism, directly interacting with MLT on HSCs[20]. In human cholangiopathies such as PBC and PSC, an initial balance between cholangiocyte apoptosis and compensatory cholangiocyte proliferation is followed by a failure in cholangiocyte proliferative capacity, and enhanced apoptosis favors evolution toward ductopenia [20].

CONCLUSION

It can be concluded from our results that MLT treatment reduced tissue and cellular lesions in the liver, inhibited lipoperoxidation and DNA damage and reduced NO levels. In addition, MLT regulated cytoprotective capacity, regulating the Nrf2 pathway and restoring the enzymes NQO1 and SOD in the livers of treated animals.

Our results suggest that MLT has potential for clinical practice, although which patients might benefit, when treatment should begin, the dosage, and treatment duration must still be determined. Thus, larger studies assessing the efficacy and safety of MLT in the long term and in the later stages of cirrhosis are required before

its clinical use can be recommended.

ARTICLE HIGHLIGHTS

Research background

Liver cirrhosis, which causes millions of deaths *per* year, is characterized by the appearance of fibrotic nodules and septa caused by chronic harmful stimuli. Oxidative damage may play a key role in the development and progression of cirrhosis. Thus, promoting the identification of new antioxidant compounds can contribute to enriching the available therapeutic arsenal.

Research motivation

Numerous studies using different experimental models have reported melatonin (MLT)'s protective effects on the liver. The antioxidant effect of MLT is related to its high solubility in lipids, which facilitates its passage through cell membranes. Thus, understanding the mechanisms involved in the protective action of MLT in cirrhosis can lead to the development of new therapeutic strategies that can lead to improved medical care.

Research objectives

The aim of the study was to evaluate the protective action of MLT in cirrhosis induced by bile duct ligation (BDL) in rats.

Research methods

Wistar rats were divided into a control group, a MLT control group, a BDL group, and a BDL group treated with MLT. Intraperitoneal administration of MLT at a dose of 20 mg/kg of body weight started on the 15th day after the beginning of the experiment and continued daily for 14 d. At the end of the experiment, the animals were euthanized. Blood was collected for liver integrity tests and the liver was collected for histological analysis, DNA damage assessment, and biochemical and Western blot analysis of proteins related to oxidative stress and the inflammatory process.

Research results

MLT promoted a significant improvement in the biochemical parameters of oxidative stress markers and the inflammatory process. DNA damage was also lower in animals treated with MLT after undergoing BDL. Tissue damage and protein expression assessed by immunohistochemistry and Western blot analysis were significantly lower in animals treated with MLT.

Research conclusions

According to the results obtained in the evaluated parameters, treatment with MLT reduced tissue and cell damage in the liver. Our results suggest that MLT may be of use of in patients with cirrhosis.

Research perspectives

Further studies are needed to assess the long-term efficacy and safety of MLT administration in cirrhotic patients before it can be recommended in clinical practice.

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World J Gastroenterol 2022 January 21; 28(3): 365-380

DOI: 10.3748/wjg.v28.i3.365

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Case Control Study

Microbiome changes in the gastric mucosa and gastric juice in different histological stages of *Helicobacter pylori*-negative gastric cancers

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Author contributions: Ding SG, Shi YY, and Sun QH designed the study; Sun QH analyzed the data and wrote the manuscript; Sun QH, Zhang J, Fu WW and Zhang J collected the samples and conducted the laboratory experiments; all authors approved the final version of the article.

Institutional review board statement: The studies involving human participants were reviewed and approved by the Peking University Third Hospital Medical Ethics Committee (No. IRB00006761-M2017414).

Conflict-of-interest statement: No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: The

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Abstract

BACKGROUND

The gastric microbiota in patients with gastric cancer (GC) has received increasing attention, but the profiling of the gastric microbiome through the histological stages of gastric tumorigenesis remains poorly understood, especially for patients with *Helicobacter pylori*-negative GC (HPNGC).

AIM

To characterize microbial profiles of gastric mucosa and juice for HPNGC carcinogenesis and identify distinct taxa in precancerous lesions.

METHODS

The 16S rRNA gene analysis was performed on gastric mucosa from 134 *Helicobacter pylori*-negative cases, including 56 superficial gastritis (SG), 9 atrophic gastritis (AG), 27 intestinal metaplasia (IM), 29 dysplasia (Dys), and 13 GC cases, to investigate differences in gastric microbial diversity and composition across the disease stages. In addition, paired gastric mucosa and juice samples from 18 SG, 18 IM, and 18 Dys samples were analyzed. α-Diversity was measured by Shannon and Chao1 indexes, and β-diversity was calculated using partial least squares discrimination analysis (PLS-DA). Differences in the microbial composition across disease stages in different sample types were assessed using the linear discriminant analysis effect size.

RESULTS

The diversity and composition of the bacterial microbiota in the gastric mucosa changed progressively across stages of gastric carcinogenesis. The diversity of the

authors have read the ARRIVE Guidelines, and the manuscript was prepared and revised according to the ARRIVE Guidelines.

Supported by National Natural Science Foundation of China, No. 81700496, and No. 81870386; Peking University Medicine Fund of Fostering Young Scholars' Scientific & Technological Innovation, No. BMU2021PY002; and Key Laboratory for Helicobacter pylori Infection and Upper Gastrointestinal Diseases, Beijing Key Laboratory, No. BZ0371.

Country/Territory of origin: China

Specialty type: Gastroenterology and hepatology

Provenance and peer review: Unsolicited article; externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification
Grade A (Excellent): A
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

Received: November 26, 2021
Peer-review started: November 26, 2021
First decision: December 12, 2021
Revised: December 14, 2021
Accepted: January 11, 2022

gastric mucosa microbiota was found to be significantly lower in the IM and Dys groups than in the SG group, and the patients with GC had the lowest bacterial community richness ($P < 0.05$). Patients with IM and those with Dys had similar gastric mucosa microbiota profiles with *Ralstonia* and *Rhodococcus* as the predominant genera. Microbial network analysis showed that there was increasing correlation strength between IM and Dys ($|correlation| \geq 0.5$, $P < 0.05$). GC and its precancerous lesions have distinguishable bacterial taxa; our results identified HPNGC-associated bacteria *Streptococcaceae* and *Lactobacillaceae* ($P < 0.05$). Additionally, across precancerous lesion stages from AG to Dys in *Helicobacter pylori*-negative patients, *Burkholderiaceae* abundance continuously increased, while *Streptococcaceae* and *Prevotellaceae* abundance presented a continuous downward trend. Furthermore, the microbial diversity was higher in gastric juice ($P < 0.001$) than in the mucosa, while PLS-DA revealed a statistically significant difference between the two groups (ANOSIM, $P = 0.001$). A significant difference in the microbial structure was identified, with *Proteobacteria* being more prevalent in the gastric mucosa and *Firmicutes* being more abundant in gastric juice.

CONCLUSION
Our results provide insights into potential taxonomic biomarkers for HPNGC and its precancerous stages and assist in predicting the prognosis of IM and Dys based on the mucosal microbiota profile.

Key Words: Gastric mucosa; Gastric juice; Microbiota; Stomach neoplasms; Histological stages; 16s RNA gene sequencing

Core Tip: The gastric microbiome profile of *Helicobacter pylori*-negative precancerous lesions is poorly understood. This is the first study, to our knowledge, to compare the microbiota differences between paired gastric mucosa and gastric juice at different stages of gastric neoplastic progression. The findings revealed that the bacterial community of gastric juice differed from that of the gastric mucosa and that *Helicobacter pylori*-negative gastric cancer and precancerous lesions have distinct bacterial taxa. Patients with intestinal metaplasia and dysplasia had similar gastric mucosa microbiota profiles, with *Ralstonia* and *Rhodococcus* being the most predominant genera, which could aid in prognosis prediction.

Citation: Sun QH, Zhang J, Shi YY, Zhang J, Fu WW, Ding SG. Microbiome changes in the gastric mucosa and gastric juice in different histological stages of *Helicobacter pylori*-negative gastric cancers. *World J Gastroenterol* 2022; 28(3): 365-380
URL: https://www.wjgnet.com/1007-9327/full/v28/i3/365.htm
DOI: https://dx.doi.org/10.3748/wjg.v28.i3.365

INTRODUCTION

Gastric cancer (GC) is one of the most common tumor types. Despite its declining prevalence, GC is the sixth most prevalent cancer worldwide, accounting for 8.2% of all cancer-related fatalities[1]. *Helicobacter pylori* (*H. pylori*) infection is one of the major carcinogens associated with GC[2]. In the etiology of GC, *H. pylori* is the most important pathogen in the development of GC due to atrophic gastritis (AG), which mostly results in intestinal-type GC and non-AG, which primarily results in diffuse-type GC[3]. Gastric adenocarcinoma is a complex disease associated with several different risk factors. Approximately 30% of stomach malignancies are not caused by *H. pylori* infection[4]. Heterogeneity is influenced by factors such as demographic characteristics, lifestyle, excessive salt and nitrate diet, race, and genetic variables[5-9].

Even though *H. pylori* is recognized as a class I carcinogen by the International Agency for Research on Cancer because of its association with GC, an *H. pylori*-

Article in press: January 11, 2022
Published online: January 21, 2022

P-Reviewer: Huh YM, Hwang GS, Shin CM
S-Editor: Liu JH
L-Editor: A
P-Editor: Liu JH

negative subgroup does exist[10]. The proportion of *H. pylori*-negative GC (HPNGC) among patients with GC varies from 0.7% to 47.8% in previous reports, and a possible poorer prognosis might exist in HPNGC[11-14].

It is gradually accepted that the stomach does indeed host a robust microbiota due to breakthroughs in PCR and metagenomics methods[15]. An increasing number of studies on the link between the gastric microbiota and GC have been spurred by these technological advancements. The majority of GC cases are the intestinal type of non-cardia GC, which develops from AG to intestinal metaplasia (IM) and to GC *via* predictable progression[16]. Gastric microbiota diversity has been characterized by the severity of phenotypes, including SG, AG, IM, and GC, in many studies[15,17-19].

However, it remains unclear whether there is a correlation between the diversity of gastric microbiota and the development of gastric carcinogenesis. There is currently no consensus on the relationship between microbiota diversity and GC development stage, despite the fact that several studies have used similar methods of data collection, exclusion criteria, molecular methods for analysis, and similar measures for diversity (via Shannon's diversity index or Chao1 richness estimator). The majority of studies investigating this problem have used gene sequencing on mucosal biopsy samples collected by upper endoscopy to examine the gastric microbiota of patients with conditions ranging from normal gastric mucosa to GC[17,20-22].

Until recently, although the gastric microbiota in patients with GC has received increasing attention, only a limited number of studies have focused on patients with HPNGC and research on gastric juice microbiota between precancerous disease progression has remained relatively scarce. Prioritizing patients with HPNGC and analyzing gastric juice samples will help fill the gap in our understanding of GC. Therefore, our study focused on *H. pylori*-negative patients and performed 16S rRNA gene analysis of gastric mucosal and juice samples to determine gastric microbiome dysbiosis across stages of HPNGC and the differences in bacterial communities between gastric mucosa and juice.

MATERIALS AND METHODS

Study design and participants

Patients were recruited from the Department of Gastroenterology of Peking University Third Hospital between September 2019 and October 2020 during upper gastroenterology endoscopic examination or endoscopic submucosal dissection[A1] due to precancerous mucosal lesions. Written informed consent was obtained from all subjects in this study. This study was performed in accordance with the Declaration of Helsinki of the World Medical Association and was approved by the Peking University Third Hospital Medical Ethics Committee (No. IRB00006761-M2017414).

The inclusion criteria were as follows: (1) Age > 18 years; and (2) biopsy specimens and gastric juice. The exclusion criteria were: (1) Present use of antibiotics, antacids, probiotics, and prebiotics or within the last month before gastroscopy; (2) Previous gastric surgery; (3) Use of immunosuppressants; (4) Comorbidity and complications with serious heart, liver, lung, kidney, blood, endocrine, nervous system, or autoimmune diseases; (5) Bile reflux gastritis, gastroesophageal reflux disease, gastroduodenal or esophagus ulcer, or colorectal cancer; (6) A positive test for human immunodeficiency virus or hepatitis B or C virus; and (7) pregnancy or lactation. Experienced endoscopists performed all endoscopic examinations and obtained biopsy specimens and gastric juice. Demographic information, medical history, medication use, and dietary habits were collected from all subjects.

Sampling and histological evaluation

Gastric mucosal biopsy samples of 1-2 mm were obtained using standard gastroscopic forceps. A biopsy for histologic examination was performed based on the disease condition and as needed. The gastric biopsy samples for histological examination were fixed in 10% formalin and placed in separate vials, which were labeled according to their topographic site. Additional mucosal biopsy specimens were taken from the gastric antrum for microbial analysis. The biopsy specimens for microbial analysis were immediately frozen in liquid nitrogen, transferred to the laboratory, and stored at −80°C until DNA extraction. Gastric juice was drained in a sterile drainage tube at the beginning of the endoscopy. Then, the mucous material was removed by centrifugation at 4000 rpm [A2] for 10 min at 4°C, and samples were stored at -80°C until DNA extraction.

Two pathologists reviewed the gastric mucosa specimens separately according to the criteria proposed by the Chinese Association of Gastric Cancer[23] and the Updated Sydney System[24]. The diagnosis and classification of dysplasia (Dys) were determined using the revised Vienna Classification System[25]. GC was confirmed to have gastric adenocarcinoma and was divided into diffuse, intestinal, and mixed types according to the Lauren Classification. Each biopsy was diagnosed as non-atrophic superficial gastritis (SG), chronic AG, IM, or Dys based on the most severe histology. Improved Warthin-Starry (W-S) silver staining was performed on each gastric mucosa specimen. Both positive 13C-urea breath test and positive W-S staining identified the specimens as *H. pylori*-positive; otherwise, they were preliminarily identified as negative.

DNA extraction and 16s rRNA gene sequencing

Microbial genomic DNA was isolated using the E.Z.N.A® Soil DNA Kit (Omega Biotek, Norcross, GA, United States) according to the manufacturer's instructions. The V3-V4 hypervariable regions of the bacterial 16S ribosomal RNA gene were amplified using the primers 338 F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). The PCR cycling conditions were as follows: initial denaturation at 95°C for 3 min, followed by 27 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 45 s, and a final extension at 72°C for 10 min.

16S rRNA gene sequencing data processing

Raw reads of 16S rRNA gene sequences were de-multiplexed and quality-filtered using the Quantitative Insights Into Microbial Ecology (QIIME) platform[26]. Sequences were then clustered into OTUs based on 97% similarity. Using the Ribosomal Data Project Bayesian Classifier in QIIME, operational taxonomic units (OTUs) were assigned to phyla, classes, orders, families, and genera, and their relative abundances were calculated[27].

Bioinformatics analysis

Bioinformatics analyses were performed using the Majorbio cloud platform. The read counts were normalized using the total sum normalization. Based on the normalized OTU abundance profile, microbial alpha diversity was measured using the Shannon and Chao1 indices. Alpha diversity indices were compared by one-way analysis of variance (ANOVA) followed by false discovery rate (FDR) correction. The dissimilarity of the microbial communities among groups was evaluated by partial least squares discrimination analysis (PLS-DA) using R software. Sample clustering in beta diversity analysis was tested using analysis of similarity (ANOSIM) using the vegan package in R software. Relative bacterial abundances were analyzed using the Kruskal-Wallis test with FDR correction for multiple testing. The key bacterial genera responsible for discrimination between different groups were identified using the linear discriminant analysis (LDA) effect size (LEfSe) algorithm. LDA > 3.5 and $P < 0.05$ indicated significantly enriched microbial communities[28]. The microbiome analyst platform was used to explore and visualize the associations between the core microbes. Heatmaps were generated according to the relative abundance of taxa using R software (http://www.R-project.org).

Network analysis of core microbes

Spearman correlation analysis was performed to calculate the correlation coefficients (r values) between specific disease-related genera in the gastric mucosa. Two genera were connected by an edge if the correlation between them meets the P value ($P < 0.05$) and correlation threshold ($|\text{correlation threshold}| \geq 0.5$) cut-off. The Kruskal–Wallis test was conducted to compare the interaction strengths between the different gastric lesion groups. Statistical significance was set at $P < 0.05$.

Data analysis

Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software Inc., La Jolla, CA, United States). Data are presented as the mean ± SEM. ANOVA was used to compare differences among groups, followed by FDR correction for multiple comparisons. Statistical significance was set at $P < 0.05$. Correlation coefficients between disease phenotype parameters and alterations in microbial taxa were analyzed using Spearman's correlation analysis.

RESULTS

Demographic characteristics of study participants

A total of 183 patients were included, including 83 patients with SG, 21 with AG, 33 with IM, 34 with Dys, and 15 with GC according to the pathological report. Furthermore, samples with < 1% *H. pylori* relative abundance were grouped as *H. pylori*-negative, while those with > 1% *H. pylori* relative abundance were grouped as *H. pylori*-positive[18]. According to this standard, 56 SG, 9 AG, 27 IM, 29 Dys, and 13 GC were confirmed as *H. pylori*-negative and enrolled in our cohort. The demographic characteristics of the subjects are shown in Supplementary Table 1.

Gastric mucosa microbiota diversity

After sequencing and quality filtering, a total of 11699206 high-quality reads were generated from all samples. The average length of the sequences was 433 bp. The data were rarefied to 7234 sequences per sample to control for variations in sequencing efforts and clustered into 2296 OTUs at 97% sequence similarity. First, to test the sequencing depth, rarefaction curves were drawn, and the sequencing data volume was sufficient (Figure 1A). The generated Venn diagram showed that 103 OTUs were shared by five groups, with 489, 91, 215, 171, and 62 OTUs unique to the SG, AG, IM, Dys, and GC groups, respectively (Figure 1B). The Shannon and Chao1 indices were used to describe the α-diversity of the gastric bacterial community. The diversity and richness of the microbial community showed a declining trend across stages of gastric carcinogenesis, from SG, AG, IM, and Dys to GC. The diversity of microbiota was significantly higher in the SG group than in the IM and Dys groups (Shannon index, P = 0.003 and 0.001, respectively), and the richness of the microbiota was significantly higher in the SG group than in the GC group (Chao1 index, P = 0.027, Figure 1C). The β-diversity analysis with PLS-DA based on the OTU level revealed a pattern in which the samples were assigned into four separate groups (ANOSIM, P = 0.005; Figure 1D). Provoked by this interesting pattern, we conducted hierarchical clustering analysis at the genus level. IM samples were divided into two condensed groups, and the same result was applied to the Dys samples (Supplementary Figure 1). The IM and Dys samples were regrouped based on a hierarchical clustering tree plot. Subgroups IM-1 and Dys-1 had a similar microbiota composition with a high relative abundance of *Ralstonia* (Supplementary Figure 2).

Mucosal bacteria changes in different histological stages of gastric carcinogenesis

The differences in the gastric mucosa microbiota between each group were investigated at different taxonomic levels. The proportion of community abundance at the phylum level was calculated and is shown in Figure 2. Firmicutes, Bacteroidota, unclassified_k__norank_d__Bacteria, and Actinobacteria were the most predominant phyla, contributing to > 90% of the microbial composition of all groups. Both IM and Dys had higher abundances of Proteobacteria than the other disease stages (P < 0.001). The clusters of IM and Dys were close to each other, suggesting a similar gastric microbiota profile. Firmicutes was more abundant in patients with GC than in those with IM and Dys (P = 0.001 for both). Bacteroidetes was less abundant in the Dys group than in the SG group (P = 0.029) (Supplementary Table 2). The clusters of IM and Dys were close to each other, suggesting a similar gastric microbiota profile.

As shown by community analysis sunburst plots at the family level, *Burkholderiaceae* (12.44%), *unclassified_k__norank_d__Bacteria* (9.34%), *Prevotellaceae* (7.98%), and *Streptococcaceae* (7.54%) were more abundant in patients with SG. *unclassified_k__norank_d__Bacteria* (26.29%), *Prevotellaceae* (9.42%), *Streptococcaceae* (9.21%), and *Lactobacillaceae* (6.42%) were the main communities in patients with AG. *Burkholderiaceae* (34.08%), *Streptococcaceae* (7.94%), *Neisseriaceae* (6.16%), and *Prevotellaceae* (5.34%) were more abundant in the IM group. *Burkholderiaceae* (34.59%), *unclassified_k__norank_d__Bacteria* (4.73%), *Prevotellaceae* (4.52%), and *Streptococcaceae* (4.30%) were more abundant in patients with Dys. In the patients with GC, *Streptococcaceae* (23.92%), *Prevotellaceae* (11.11%), *Lactobacillaceae* (8.61%), and *Burkholderiaceae* (7.41%) were the dominant families. With the precancerous lesion stages from AG to Dys, *Burkholderiaceae* abundance continuously increased, while *Streptococcaceae* and *Prevotellaceae* presented a continuous trend of decline in abundance. *Streptococcaceae* and *Lactobacillaceae* abundance was significantly higher in the GC group than in the SG group (P < 0.05) (Figure 2B).

At the genus level, the top 12 genera that showed significant differences from each other were identified (Figure 2C). Taxonomic analysis indicated that the relative abundance of *Ralstonia* and *Rhodococcus* was significantly higher in patients with IM

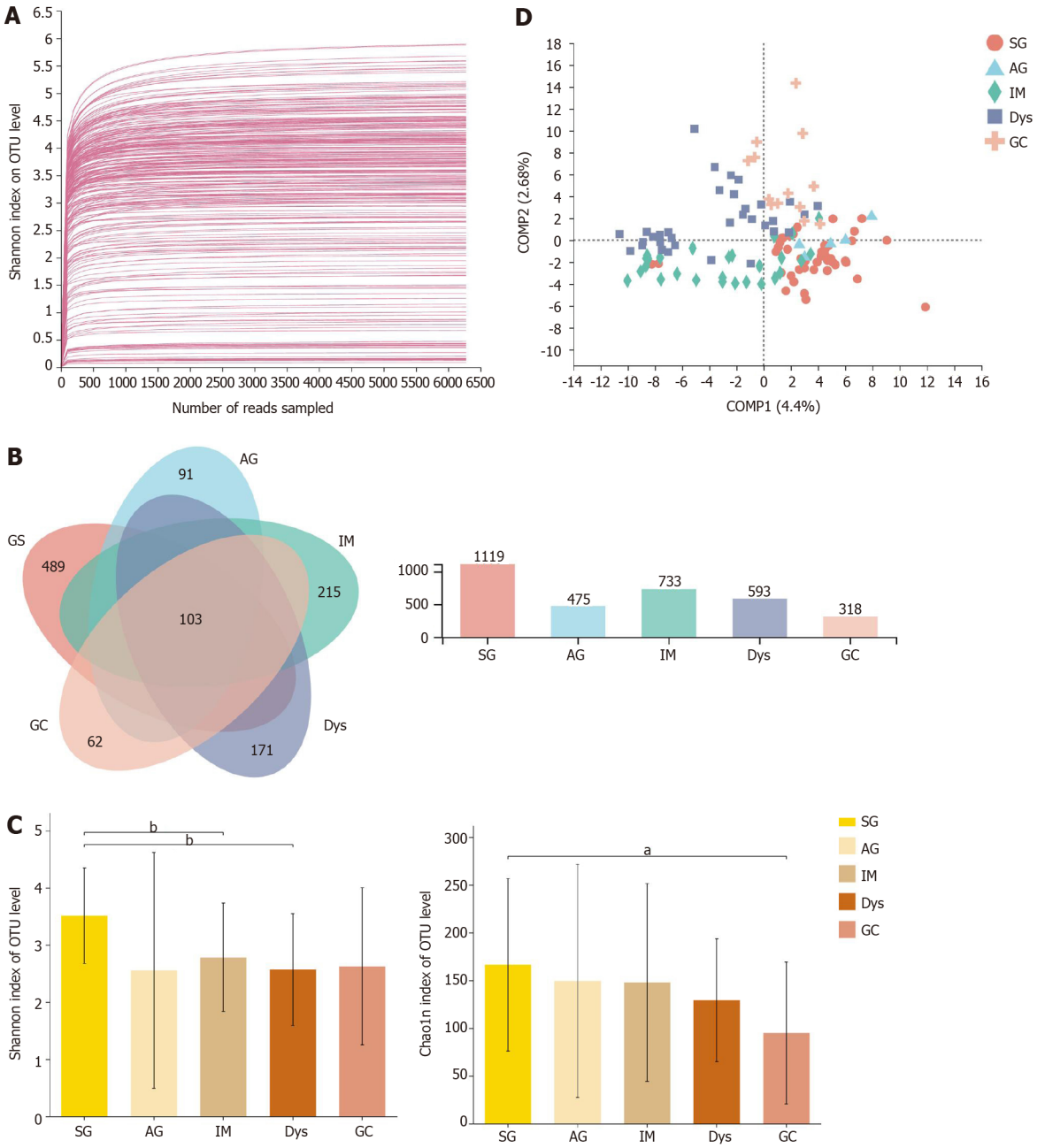

Figure 1 The microbial diversity analysis in different groups. A: Rarefaction curves of Shannon index for operational taxonomic units; B: Venn diagram; C: α-diversity indices; D: β-diversity measured by partial least squares discrimination analysis. [a]*P* < 0.05; [b]*P* < 0.01; [c]*P* < 0.001. SG: Superficial gastritis; AG: Atrophic gastritis; IM: Intestinal metaplasia; Dys: Dysplasia; GC: Gastric cancer.

and Dys than in those with SG (*Ralstonia*: $P = 0.008$ and 0.004; *Rhodococcus*: $P = 0.008$ and 0.038, respectively). *Streptococcus* and *Bifidobacterium* abundance was significantly higher in patients with GC than in those with SG ($P = 0.013$ and 0.015, respectively). *Raoultella* abundance increased in patients with Dys, and *norank_f__ mitochondria* increased in patients with AG when compared to those with SG ($P = 0.002$ and 0.008, respectively) (Table 1).

LEfSe analysis was used to identify the most relevant taxa responsible for the differences among disease stages. An LDA cutoff score of 3.5 was used to estimate the discriminatory impact of each community on the phylogenetic distribution. A total of 42 taxa were identified as key participants in the five groups (Figure 2D). Figure 2E shows the most relevant taxa responsible for the differences among disease stages at the genus level, with *Bacteroides* and *Geobacillus* identified in the SG group; *Faecalibacterium*, *Blautia*, and *norank_f__Mitochondria* in the AG group; *Rhodococcus* and *Ralstonia* in the IM group; *Enterococcus*, *Burkholderia-Caballeronia-Paraburkholderia*, and *Raoultella* in the Dys group; and *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* in the GC group (Figure 2D).

Table 1 Relative abundance of the selected top 34 genera in different histological stages

	Relative abundance (%)						
	SG	AG	IM	Dys	GC	One-way ANOVA, *P* value	*P* value
g__Ralstonia	10.43	3.84	31.05	31.04	7.97	0.000	0.008[b]; 0.004[c]
g__Streptococcus	7.52	9.19	7.91	4.28	14.90	0.028	0.013[d]
g__Prevotella	5.81	7.83	3.82	3.40	5.70	0.111	
g__Lactobacillus	4.56	6.42	1.16	2.57	9.23	0.009	
g__Neisseria	3.80	1.05	6.03	2.81	2.36	0.538	
g__Veillonella	3.15	3.00	2.31	1.49	4.53	0.497	
g__Burkholderia-Caballeronia-Paraburkholderia	1.93	1.06	2.93	3.45	1.72	0.043	
g__Haemophilus	2.95	0.78	2.08	1.32	2.33	0.540	
g__Alloprevotella	2.05	1.54	1.51	1.06	1.39	0.373	
g__Acinetobacter	1.74	0.96	1.12	1.78	1.93	0.716	
g__Actinomyces	1.42	2.80	1.04	0.92	1.22	0.364	
g__Escherichia-Shigella	1.86	1.12	1.11	1.70	1.47	0.193	
g__Fusobacterium	1.85	0.96	1.57	0.97	1.29	0.308	
g__Pseudomonas	1.56	0.47	0.88	1.65	1.70	0.128	
g__Porphyromonas	2.14	0.53	1.38	0.78	1.32	0.259	
g__Geobacillus	1.68	0.99	0.52	1.25	1.12	0.022	
g__Bifidobacterium	0.50	1.53	0.18	0.72	2.09	0.001	0.015[d]
g__Rhodococcus	0.50	0.29	2.11	1.79	0.11	0.003	0.008[b]; 0.038[c]
g__Gemella	0.93	1.18	0.63	0.32	1.39	0.119	
g__Delftia	1.07	0.35	0.95	0.90	1.04	0.609	
g__Granulicatella	0.86	0.45	1.08	0.52	1.15	0.659	
g__Bacteroides	1.49	1.08	0.22	0.54	0.66	0.003	
g__Leptotrichia	0.66	0.73	1.02	0.49	0.65	0.689	
g__norank_f__Mitochondria	0.44	2.51	0.15	0.16	0.10	0.016	0.008[a]
g__Rothia	0.77	0.70	1.00	0.28	0.56	0.762	
g__unclassified_p__Proteobacteria	0.49	1.03	0.64	0.28	0.12	0.195	
g__Raoultella	0.03	0.02	0.81	1.60	0.00	0.000	0.002[c]
g__Sphingomonas	0.54	0.17	0.33	0.56	0.81	0.742	
g__Blautia	0.83	0.86	0.18	0.23	0.32	0.001	
g__Clostridium_sensu_stricto_1	0.24	0.43	0.30	0.81	0.54	0.292	
g__TM7x	0.57	0.48	0.63	0.28	0.26	0.269	
g__Corynebacterium	0.64	0.16	0.24	0.64	0.45	0.777	
g__norank_f__norank_o__Chloroplast	0.63	0.53	0.33	0.22	0.30	0.924	
g__Faecalibacterium	0.62	0.88	0.11	0.16	0.21	0.008	

[a]SG *vs* AG.
[b]SG *vs* IM.
[c]SG *vs* Dys.
[d]SG *vs* GC. SG: Superficial gastritis; AG: Atrophic gastritis; IM: Intestinal metaplasia; Dys: Dysplasia; GC: Gastric cancer.

Associations of specific genera and their differences between stages of gastric lesions

The relative abundance of the same 13 genera (the most relevant taxa responsible for the differences among disease stages are presented in Figure 2D) was compared among different gastric lesion groups. A network diagram was drawn based on the correlation between the genera to reflect the interactions between samples. The sizes of the nodes in the figure indicate the abundance of genera. The red color indicates a positive correlation, and green indicates a negative correlation. The thicker the line, the stronger the correlation between the genera. These results were used to visualize and identify possible associations among the important taxa. The IM and Dys groups had more complex interactions than the SG and GC groups (Figure 3). The transitivity, diameter, and average shortest path length of the different histological stages are shown in Supplementary Table 3.

The bacterial community of gastric juice was different from that of gastric mucosa

Paired-gastric juice and mucosa from 18 SG, 18 IM, and 18 Dys patients were analyzed. In the first step, we analyzed the gastric juice from patients with SG, IM, and Dys. The richness of the microbiota was significantly higher in the SG group than in the Dys group (Chao1 index, $P = 0.025$), but there were no significant differences in the diversity of microbiota between these groups (Figure 4A). Although β-diversity analysis with PLS-DA based on the OTU level revealed a pattern with three clusters, ANOSIM showed that the clusters for the three groups were not significantly different ($P = 0.230$; Figure 4B). As shown by ternary analysis at the family level, *Burkholderiaceae* was more abundant in the IM group, *Fusobacteriaceae* and *Prevotellaceae* were more abundant in the SG group, and *Veillonellaceae* and *Staphylococcaceae* were more abundant in the Dys group (Figure 4C). At the genus level, taxonomic analysis indicated that the relative abundance of *Alloprevotella* in Dys was significantly decreased, while that in IM and *Campylobacter* abundance in SG were significantly increased ($P < 0.05$, Figure 4D).

Next, the microbial α-diversity and β-diversity were measured to analyze the differences in the microbiota structure between the gastric mucosa and juice. We found that the microbial community diversity was significantly higher in gastric juice ($P < 0.001$), while there was no significant difference in microbial community richness between the two groups (Figure 5A). PLS-DA at the OTU level revealed a statistically significant separation of the groups (ANOSIM, $P = 0.001$; Figure 5B), suggesting different microbial community structures. *Proteobacteria* (59.30%), *Firmicutes* (14.37%), and *Bacteroidetes* (7.94%) were three of the most predominant phyla in the gastric mucosa, while *Firmicutes* (38.86%), *Proteobacteria* (20.01%), and *Bacteroidetes* (17.33%) were the top three most abundant phyla in gastric juice (Figure 5C).

To assess the microbiota characteristics of different stomach microhabitats, we compared pairs of gastric juice and mucosa samples for each disease stage from patients with SG, IM, and Dys. LEfSe analysis was applied to identify the most relevant taxa responsible for the differences between gastric liquid and mucosa among the disease stages (Figure 6A). We focused on bacterial taxa with different abundances at the genus and species levels. In the gastric juice of patients with Dys, enrichment in the genera *unclassified_o__Lactobacillales* and *Veillonella* was observed. In the gastric mucosa group of patients with Dys, the enriched genera were *Raoultella* and *Bacteroides*. The SG-enriched genera in the gastric mucosa were *Escherichia-Shigella* and *norank_f_Mitochondria* (Figure 6B).

DISCUSSION

In recent years, many researchers and clinicians have explored the role of the microbiome in various disease processes, which has resulted in a significant surge in the number of studies on this topic[29]. Although the severely acidic conditions of the stomach have formerly hampered research into the gastric microbiota, studies on the gastric microbiota have risen over the past decade owing to the development of modern PCR techniques and metagenomic analyses. The majority of research has compared the gastric mucosal microbiota of GC to that of SG or healthy controls without distinguishing non-*H. pylori*-infected individuals from *H. pylori*-infected ones [30-32]. Additionally, the number of studies examining the gastric microbiota utilizing gastric juice samples is still limited, and well-designed comparative studies analyzing the link between the mucosal and luminal microbiota are even rarer. To bridge these gaps, we studied the mucosal bacterial community from SG, AG, IM, Dys, and GC in

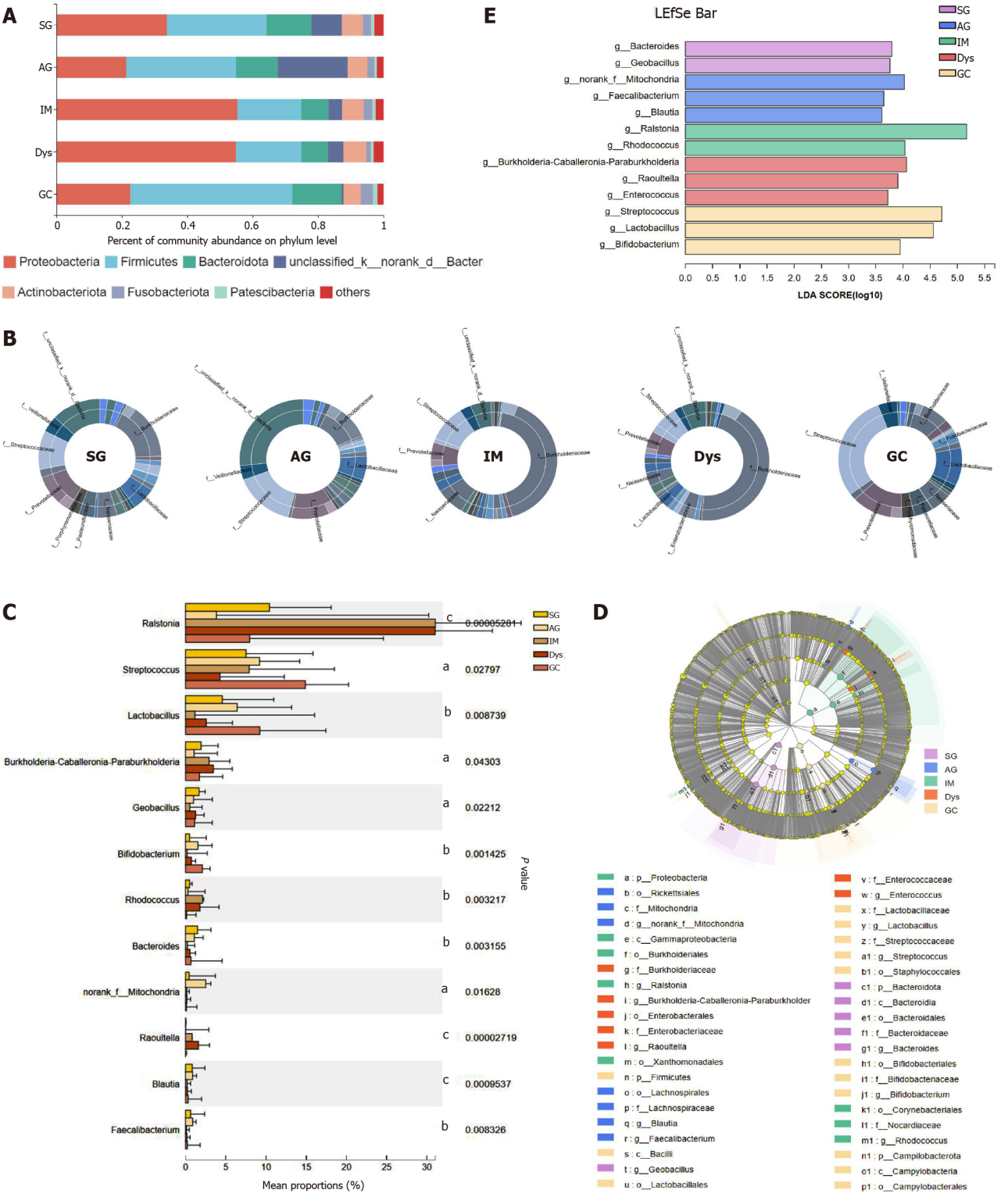

Figure 2 The mucosa microbiota composition in different groups. A: Relative abundance of phyla in five groups; B: Community analysis sunburst plot on family level; C: Changes in the gastric mucosa microbiota from superficial gastritis, through atrophic gastritis, intestinal metaplasia, dysplasia to gastric cancer; D: Linear discriminant analysis effect size (LEfSe) analysis from phylum to genus; E: Histogram of LEfSe analysis at the genus level. Significance was obtained by LEfSe (Kruskal–Wallis test) at $P < 0.05$, and linear discriminant analysis score>3.5. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$. LDA: Linear discriminant analysis; LEfSe: LDA effect size; SG: Superficial gastritis; AG: Atrophic gastritis; IM: Intestinal metaplasia; Dys: Dysplasia; GC: Gastric cancer.

H. pylori-negative patients as well as the bacterial composition of gastric juice and its deviations from the mucosal microbiota.

In this study, we discovered that the α-diversity of the gastric mucosa microbiota was significantly lower in the IM and Dys groups than in the SG group using the Shannon index, and that the bacterial community richness was lowest in the patients with GC, which is supported by earlier research results[19,30,32].

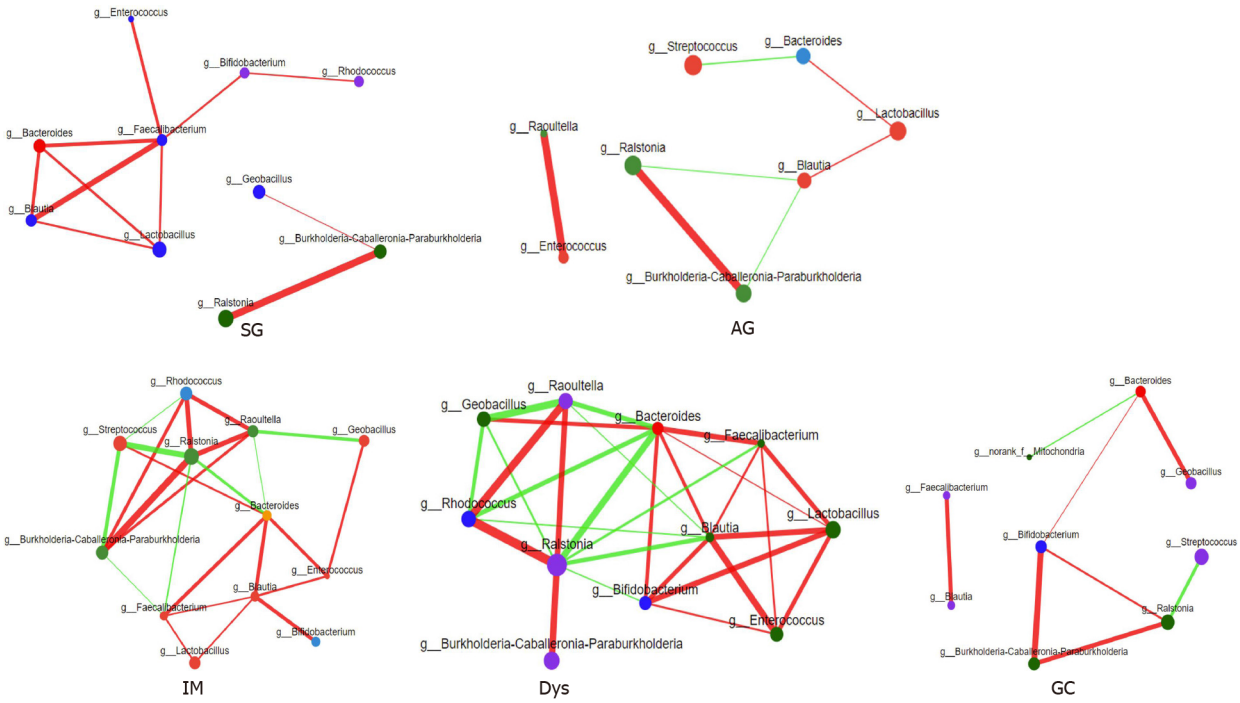

Figure 3 Correlation analysis between core microbes. SG: Superficial gastritis; AG: Atrophic gastritis; IM: Intestinal metaplasia; Dys: Dysplasia; GC: Gastric cancer.

Our study's findings on microbial β-diversity revealed that the SG, IM, Dys, and GC groups could be distinguished from each other, which is in agreement with the results of previous studies showing that there is a shift in the composition of the microbial community along different histological phases of stomach neoplastic progression[18,33]. Notably, the AG group was intermingled with the SG group in the β-diversity plot (Figure 1D), indicating that the microbial profile of AG may be comparable to that of SG. Another possibility is that a comprehensive assessment of the microbiome in AG is not feasible owing to the sample size constraint. Another interesting finding was that patients with IM were dispersed throughout the SG and Dys as a bridge in the PLS-DA (Figure 1D). Similarly, the distribution region of β-diversity in the Dys group exhibited distinct characteristics: one portion heavily overlapped with the GC group, while the other part was near the IM group. Based on the above findings, we further plotted hierarchical clustering trees and found that there were two distinct clusters in the IM and Dys groups. We then investigated the bacterial composition of patients with IM and Dys and discovered that both groups could be divided into two sub-groups that corresponded to their β-diversity distribution (Supplementary Figure 1).

As precancerous lesions, IM and Dys have been considered intermediate stages between cancer and gastritis, and consecutive alterations in the microbiota may play a role in the progression of mucosal precancerous lesions. In clinical practice, it is challenging for digestive endoscopists to choose the appropriate interval and frequency of endoscopic follow-up for patients with IM or Dys. According to our findings, one possible solution to this problem is to use the microbiota profile of the gastric mucosa to determine whether a patient has a more cancer- or gastritis-like microbiota, allowing cancer-like patients to undergo more rigorous and frequent endoscopic monitoring or magnifying endoscopy because they may be at a higher risk of cancer. It might be more reasonable to assess gastric lesions by pathological reports combined with gastric microbiota profiles. Further research with a larger sample size is needed to validate this theory.

Streptococcus was found to be significantly more abundant in patients with GC than in those with SG, which is supported by the findings of multiple studies that used mucosal samples[33,34]. Interestingly, several investigations have indicated a large increase in *Streptococcus* abundance in malignant tissues compared to normal tissues from the same patients with GC, and similar findings have also been drawn using fecal samples, which made us more certain that *Streptococcus* should play an essential role throughout the process of gastric cancerization[35-37]. One plausible explanation is that *Streptococcus* antigens might induce cancer, since *Streptococcus bovis* has previously been shown to have such an effect in two studies using animal models[38,39].

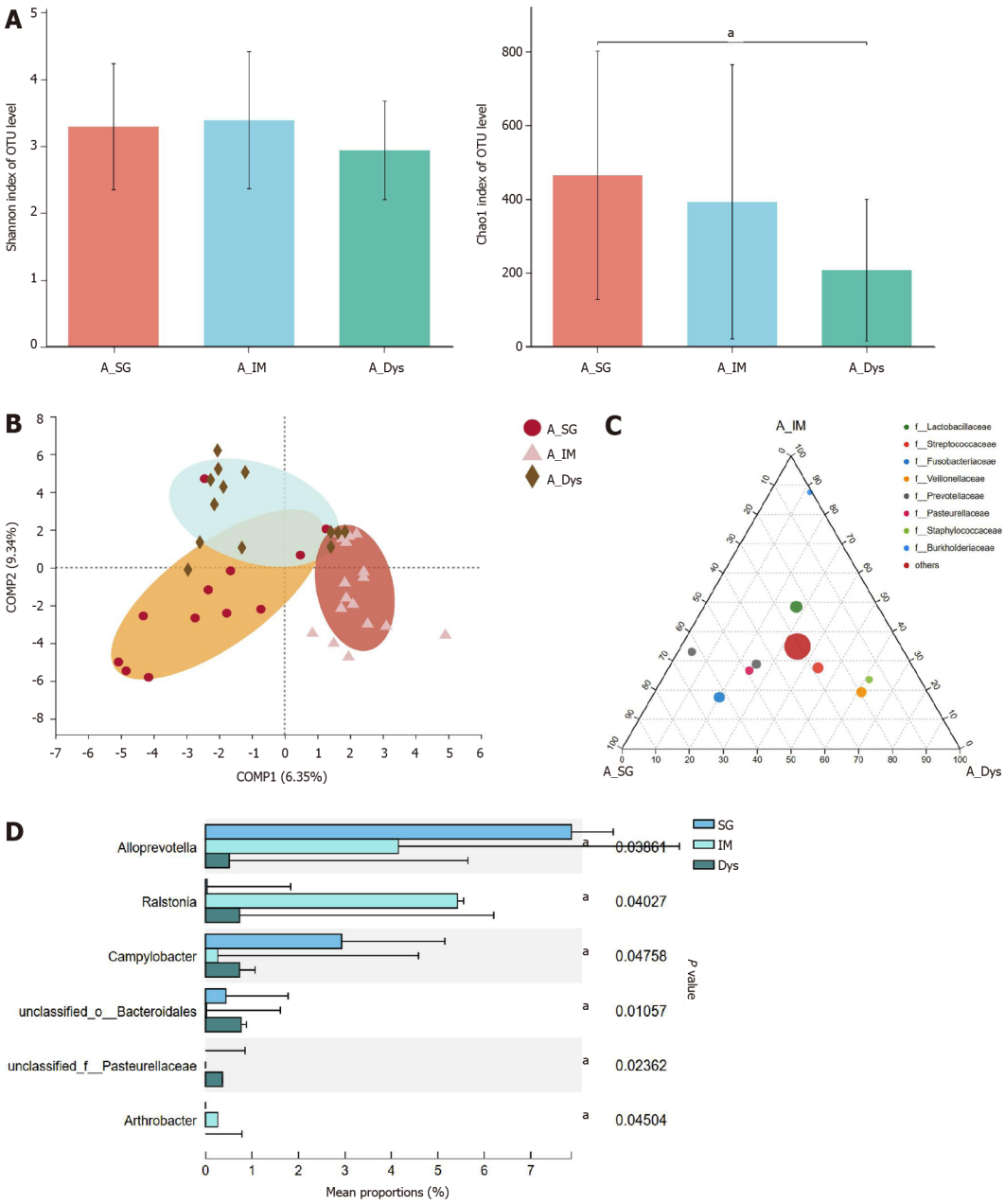

Figure 4 The microbial diversity analysis and microbiota composition of gastric juice in different groups. A: α-diversity indices; B: β-diversity measured by partial least squares discrimination analysis; C: Ternary analysis at the family level; D: Taxonomic analysis at the genus level. $^{a}P < 0.05$. SG: Superficial gastritis; IM: Intestinal metaplasia; Dys: Dysplasia.

In our study, the relative abundance of *Alloprevotella* was shown to decline significantly lower in patients with Dys than in those with SG at the genus level ($P < 0.05$, Figure 4D). This result is in-line with that of a recent study, which indicated that *Alloprevotella* levels are significantly lower in the IM/DYS group than in the normal/SG group[40]. *Alloprevotella* is known to have anti-inflammatory properties, which may explain this outcome to some degree[41,42]. In addition, *Ralstonia* abundance was found in our study to be significantly increased in the IM and Dys groups compared to in the SG group, which is consistent with the results of an earlier study[43]. *Ralstonia* has been shown to play a role in the initiation of inflammation, which explains why there was an increase in relative abundance[44]. In addition, *Ralstonia* and *Helicobacter* were verified as the top two genera of discriminant abundance in the stomachs of patients with GC, which warrants deeper analysis of the association between these two genera and GC[45].

In most clinical trials, intragastric bacterial overgrowth is examined using gastric juice culture and rarely *via* gastric mucosal tissue. Gastric juice samples are easier to collect, generally non-invasive compared to mucosal tissues, and exhibit integrated properties. They have been used to characterize the gastric microbiota in some studies

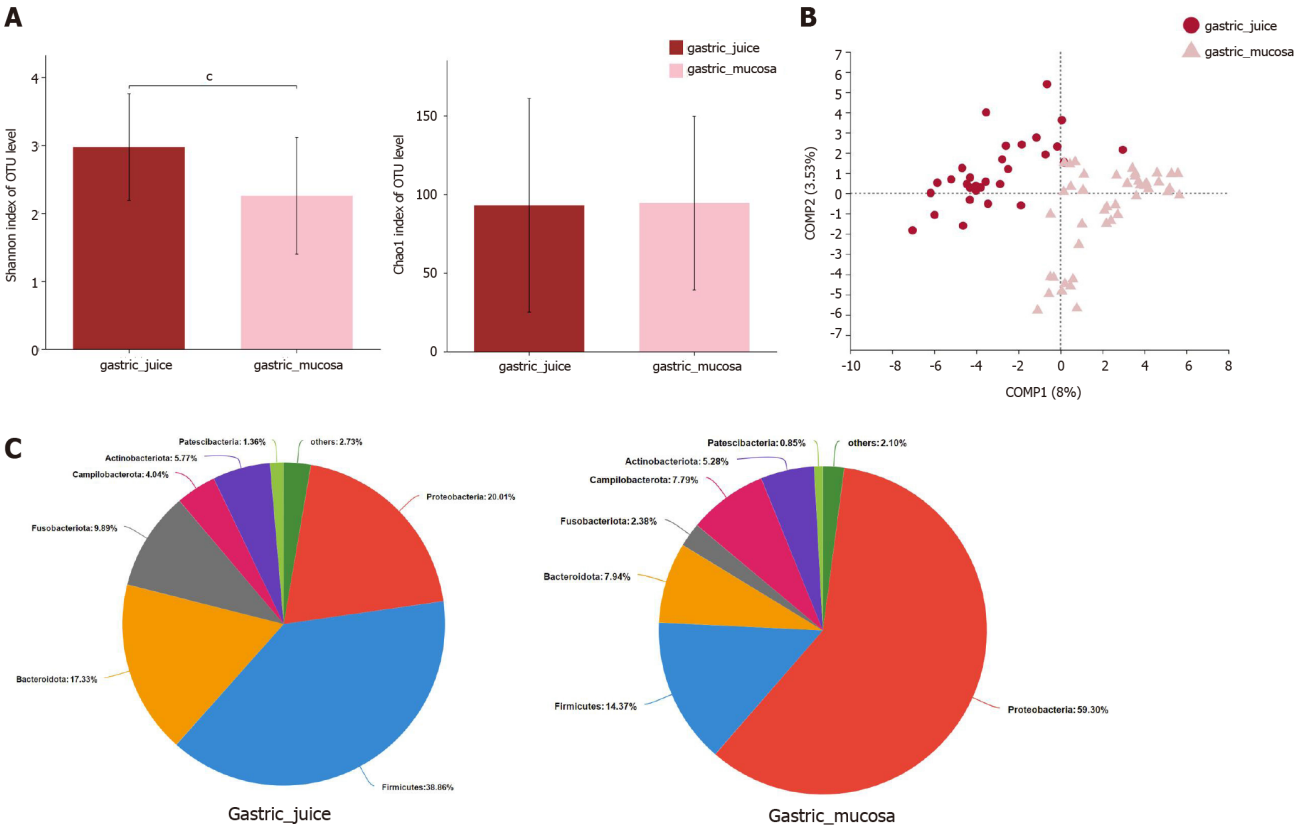

Figure 5 The microbial diversity and microbiota composition in different groups. A: α-diversity indices; B: β-diversity measured by partial least squares discrimination analysis; C: Relative abundance of phyla in two groups. °P < 0.001; SG: Superficial gastritis; AG: Atrophic gastritis; IM: Intestinal metaplasia; Dys: Dysplasia; GC: Gastric cancer.

[46,47]. In general, gastric juice samples include a combination of mucosal microbes and luminal communities[48], which have not been previously assessed in patients with GC. It has been demonstrated that oral or fecal commensal flora are usually found in the gastric juice of patients with GC[22], which indicates that there might be differences between the microbiota in gastric juice and mucosa. With respect to the influence of sample type, it was demonstrated in our study that the alterations of microbiota in gastric mucosa and gastric juice showed a discrepancy despite several earlier studies showing that microbial communities of different anatomical gastric positions are similar[18,20,36], which illustrated that gastric sample type may be a factor influencing research results, and that juice and mucosal samples should be treated separately. Future studies are still needed to confirm the differences between the mucosal microbiota and the gastral cavity microbiota.

It is a pity that only gastric juice samples in SG, IM, and Dys groups were available when samples were collected with a lack of data from the GC group. Another shortcoming is that the sample size of the AG groups was relatively small, which might not reflect the bacterial composition to the fullest. It has also been shown that tea drinking as well as fresh vegetable and fruit intake might play a role in slowing carcinogenic progression[49], which might have some influence on gastric microbiota. A detailed dietary questionnaire would ensure more rigorous and well-founded results. In-depth research on the pathogenic mechanisms of non-*H. pylori* bacteria in gastric carcinogenesis will be strongly desired in the future.

CONCLUSION

Our study showed a shift in the gastric microbial community structure along the SG-AG-IM-Dys-GC stages in the *H. pylori*-negative stages. The diversity and composition of the gastric mucosal microbiota altered gradually across the stages of gastric neoplastic progression. Patients with IM and Dys had similar gastric mucosa microbiota profiles, and their potential to be indicators of IM and Dys prognosis needs to be verified in further studies. Our findings also revealed that the bacterial

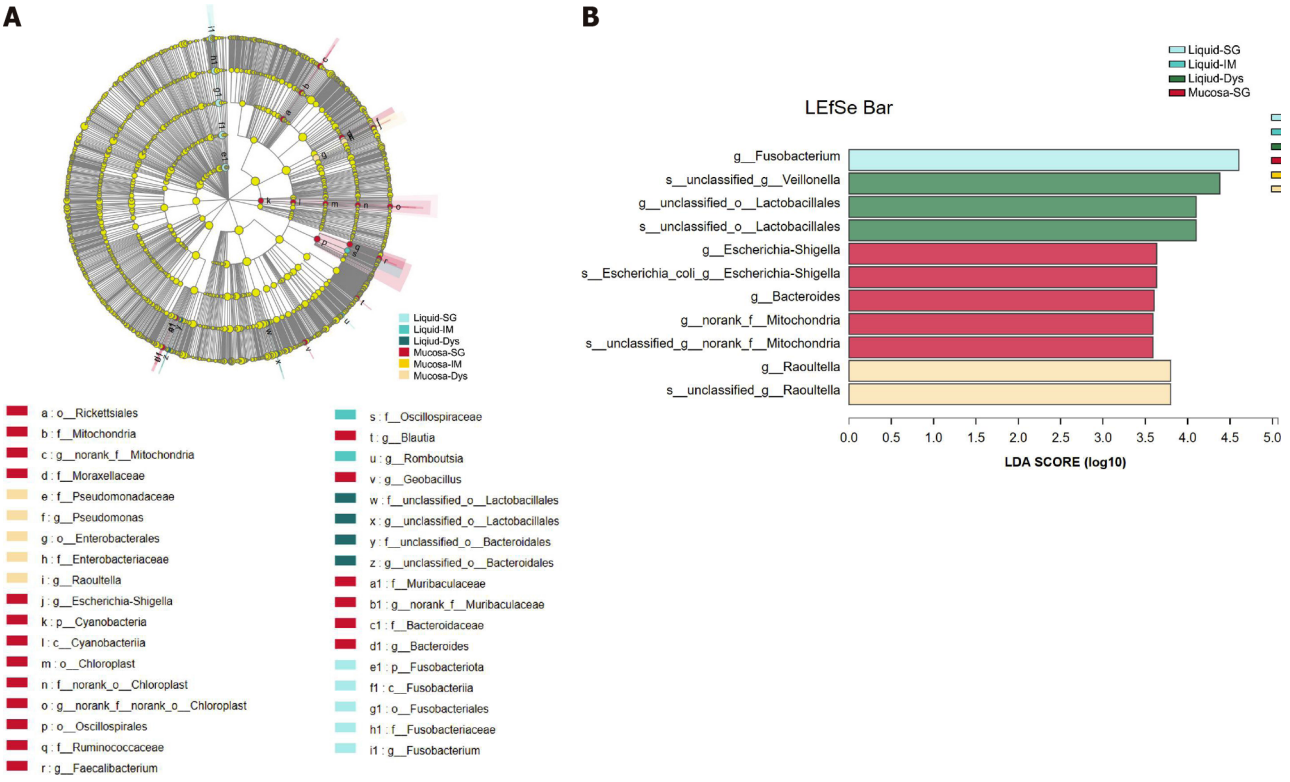

Figure 6 Linear discriminant analysis scores for differentially abundant taxonomic features among six groups. Significance was obtained by linear discriminant analysis effect size (LEfSe) (Kruskal-Wallis test) at $P < 0.05$, and linear discriminant analysis score > 3.5. A: LEfSe analysis from phylum to genus; B: Histogram of LEfSe analysis at the genus level. LDA: Linear discriminant analysis; LEfSe: LDA effect size; SG: Superficial gastritis; AG: Atrophic gastritis; IM: Intestinal metaplasia; Dys: Dysplasia; GC: Gastric cancer.

community of gastric juice differed from that of the gastric mucosa, and that HPNGC and its precancerous lesions have distinct bacterial taxa. *Streptococcaceae* and *Lactobacillaceae* were enriched in HPNGC. In addition, from AG to Dys, *Burkholderiaceae* abundance increased continuously, while *Streptococcaceae* and *Prevotellaceae* presented a continuous downward trend in abundance, which suggested that *Burkholderiaceae*, *Streptococcaceae*, and *Prevotellaceae* might play different roles in the carcinogenesis of HPNGC.

ARTICLE HIGHLIGHTS

Research background

The gastric microbiome through the histological stages of gastric tumorigenesis remains poorly understood, especially for the *Helicobacter pylori*-negative gastric cancer (HPNGC).

Research motivation

To get a better knowledge of gastric microbiota and to identify microbial indicators at different histological stages of gastric tumorigenesis.

Research objectives

To identify distinct taxa in precancerous lesions and describe microbial profiles of gastric mucosa and juice for HPNGC carcinogenesis.

Research methods

We designed a clinical cohort study and utilized the 16S rRNA gene sequencing analysis.

Research results

Our study showed a change in the gastric microbial community structure along the

precancerous lesions in the *Helicobacter pylori*-negative stages. Patients with intestinal metaplasia and dysplasia had similar gastric mucosa microbiota profiles, and their potential to be indicators for prognosis. Our findings revealed that the bacterial community of gastric juice differed from that of the gastric mucosa, and that HPNGC and its precancerous lesions have distinct bacterial taxa.

Research conclusions

Using the gastric microbiota profile, we were able to identify possible taxonomic biomarkers for HPNGC and its precancerous phases, as well as help predict prognoses for intestinal metaplasia and dysplasia.

Research perspectives

Our research revealed the core pathogenic bacteria in *Helicobacter pylori*-negative precancerous lesions, allowing for further investigation of the pathogenic process.

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World J Gastroenterol 2022 January 21; 28(3): 381-398

DOI: 10.3748/wjg.v28.i3.381

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Retrospective Cohort Study

Postoperative mortality and morbidity after D2 lymphadenectomy for gastric cancer: A retrospective cohort study

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Author contributions: Brisinda G and Chiarello MM designed the research; Brisinda G, Chiarello MM and Vanella S performed the research; Crocco A and Vanella S analyzed the data; Brisinda G, Chiarello MM, Vanella S, Adams NJ and Fransvea P wrote the paper.

Institutional review board statement: Not necessary for this type of publication.

Clinical trial registration statement: Research Registry ID: researchregistry6939, https://www.researchregistry.com/browse-the-registry#home/.

Informed consent statement: Informed consent was waived due to the retrospective nature of this study.

Conflict-of-interest statement: No conflict of interest.

Data sharing statement: All the

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Abstract

BACKGROUND

Surgery for gastric cancer is a complex procedure and lymphadenectomy is often mandatory. Postoperative mortality and morbidity after curative gastric cancer surgery is not insignificant.

AIM

To evaluate the factors determining mortality and morbidity in a population of patients undergoing R0 resection and D2 lymphadenectomy for gastric cancer.

METHODS

A retrospective analysis of clinical data and pathological characteristics (age, sex, primary site of the tumor, Lauren histotype, number of positive lymph nodes resected, number of negative lymph nodes resected, and depth of invasion as

data used are present in the text. No additional data are available.

STROBE statement: The authors have read the STROBE Statement-checklist of items, and the manuscript was prepared and revised according to the STROBE Statement-checklist of items.

Country/Territory of origin: Italy

Specialty type: Gastroenterology and hepatology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification
Grade A (Excellent): A
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

Received: October 4, 2021
Peer-review started: October 4, 2021
First decision: November 7, 2021
Revised: November 9, 2021
Accepted: January 11, 2022
Article in press: January 11, 2022
Published online: January 21, 2022

P-Reviewer: An T, Rahimi-Movaghar E
S-Editor: Wang LL
L-Editor: Filipodia
P-Editor: Wang LL

defined by the standard nomenclature) was conducted in patients with gastric cancer. For each patient we calculated the Kattan's score. We arbitrarily divided the study population of patients into two groups based on the nomogram score (< 100 points or ≥ 100 points). Prespecified subgroups in these analyses were defined according to age (≤ 65 years or > 65 years), and number of lymph nodes retrieved (≤ 35 lymph nodes or > 35 lymph nodes). Uni- and multivariate analysis of clinical and pathological findings were performed to identify the factors affecting postoperative mortality and morbidity.

RESULTS
One-hundred and eighty-six patients underwent a curative R0 resection with D2 lymphadenectomy. Perioperative mortality rate was 3.8% (7 patients); a higher mortality rate was observed in patients aged > 65 years (P = 0.002) and in N+ patients (P = 0.04). Following univariate analysis, mortality was related to a Kattan's score ≥ 100 points (P = 0.04) and the presence of advanced gastric cancer (P = 0.03). Morbidity rate was 21.0% (40 patients). Surgical complications were observed in 17 patients (9.1%). A higher incidence of morbidity was observed in patients where more than 35 lymph nodes were harvested (P = 0.0005).

CONCLUSION
Mortality and morbidity rate are higher in N+ and advanced gastric cancer patients. The removal of more than 35 lymph nodes does not lead to an increase in mortality.

Key Words: Gastric cancer; Total gastrectomy; Subtotal gastrectomy; Lymphadenectomy; Kattan's nomogram; Mortality; Postoperative complications; Postoperative pancreatic fistula; Hemoperitoneum; Anastomotic leakage

Core Tip: Surgery for gastric cancer is a complex procedure. The aim of our study is to evaluate the factors determining mortality and morbidity in 186 patients undergoing R0 resection and D2 lymphadenectomy for gastric cancer. Perioperative mortality rate was 3.8%; a higher mortality rate was observed in patients aged > 65 years and in N+ patients. Mortality was related to a Kattan's score ≥ 100 points and the presence of advanced gastric cancer. Morbidity rate was 21.0%. Surgical complications were observed in 17 patients. A higher incidence of morbidity was observed in patients where more than 35 lymph nodes were harvested.

Citation: Brisinda G, Chiarello MM, Crocco A, Adams NJ, Fransvea P, Vanella S. Postoperative mortality and morbidity after D2 lymphadenectomy for gastric cancer: A retrospective cohort study. *World J Gastroenterol* 2022; 28(3): 381-398
URL: https://www.wjgnet.com/1007-9327/full/v28/i3/381.htm
DOI: https://dx.doi.org/10.3748/wjg.v28.i3.381

INTRODUCTION

Although the incidence of gastric cancer is steadily declining, the disease remains the second leading cause of cancer death[1,2]. Currently, surgery is the only potentially curative treatment for gastric cancer[3,4]. The depth of primary tumor invasion, lymph node involvement, and distant metastasis are the major predictors of prognosis for patients with gastric cancer[5].

Node metastases occur during the early stages of the disease, and lymphadenectomy is recommended as the main intervention of a radical surgical treatment[4,6,7]. According to the TNM staging system proposed by the Union for International Cancer Control (UICC) and the American Joint Committee on Cancer (AJCC)[8], the N stage is classified into 5 Levels based on the number of metastatic lymph nodes. However, the extent of lymphadenectomy, which aims to achieve the highest optimal outcome, has been a controversial topic for a long time with no worldwide consensus

as of yet[9]. A minimum of 16 lymph nodes has been recommended as an adequate number in radical gastrectomy for gastric cancer to ensure reliable N staging. Studies have shown that the number of dissected metastatic lymph nodes influences prognosis [10].

Gastric cancer surgery is a complex procedure; in this context, lymphadenectomy is mandatory[11-14]. Mortality and morbidity after curative gastric cancer surgery are not negligible[15-17]. There are many clinical and pathological factors that induce an increase in mortality and morbidity[18]. The extent of the lymphadenectomy is one of these factors. The development of postoperative complications, and the associated mortality, is also influenced by the stage of the disease, the number of lymph node metastases, the removal of contiguous organs and the age of the patient.

In this paper, we evaluated patients with histologically confirmed gastric adenocarcinoma, who underwent curative gastrectomy and D2 lymphadenectomy according to the Japanese Gastric Cancer Association (JGCA) guidelines[19,20]. The primary endpoint of the study is to evaluate the factors determining mortality and morbidity in a population of patients undergoing R0 resection and D2 lymphadenectomy for gastric cancer. For each patient we calculated the Kattan's score. In agreement with the original report by Kattan *et al*[21] the following prognostic variables were assembled for use in validating the nomogram: age, sex, primary site [distal one-third, middle one-third, proximal one-third, and gastroesophageal junction (GEJ)], Lauren histotype (diffuse, intestinal, mixed), number of positive lymph nodes resected, number of negative lymph nodes resected and depth of invasion as defined by the standard nomenclature. We arbitrarily divided the study population of patients into two groups based on the nomogram score (< 100 points or ≥ 100 points). Prespecified subgroups in these analyses were defined according to age (≤ 65 years or > 65 years) and number of lymph nodes retrieved (≤ 35 lymph nodes or > 35 lymph nodes). The cut off was used in this study since age > 65 years is considered a significant risk factor for postoperative complications in gastric surgery, and was also in accordance with a definition of age limits for elderly patients. Clinical factors and pathological findings were evaluated to identify the factors that induce increased postoperative mortality and morbidity in patients undergoing R0 surgery. Treatment factors were also analyzed for their impact on mortality and morbidity.

MATERIALS AND METHODS

This is a retrospective study. An analysis of clinical data and pathological characteristics was conducted on patients with gastric cancer observed and treated at the General Surgery Operative Unit, Fondazione Policlinico Universitario "A Gemelli" IRCCS, from January 2010 to December 2015, and at the General Surgery Operative Unit, San Giovanni di Dio Hospital, Azienda Sanitaria Provinciale Crotone, from January 2016 to June 2020.

All patients provided written consent before the surgical procedures. Preliminary approval to use patient data was obtained from the Institutional Review Board. This study was conducted according to the STROBE guidelines[22].

Inclusion criteria

Patients with histologically documented gastric cancer were included in the study. All patients underwent a complete clinical evaluation, including laboratory tests, with complete blood cell count and serum chemistry. In all patients, a preoperative staging of the neoplasm was performed. This included upper digestive endoscopy with biopsy, chest X-ray, liver ultrasound and abdomino-pelvic CT-scan. Tumors were staged according to the latest version of the pathologic classification (pTNM) of the International UICC. The histological classification followed the Lauren criteria[23].

Exclusion criteria

Gastric stump and linitis plastica type tumors were excluded from the analysis. Patients with squamous cell cancer or stromal tumors and patients in preoperative neoadjuvant treatment protocols were also excluded from the analysis. Patients with positive surgical resection margins, patients with peritoneal carcinomatosis and/or patients with metastatic disease, and patients with > 1 missing data were not included in the study.

Surgical rules

Gastrectomy is defined by the removal of the greater and lesser omentum and

perigastric lymph nodes (N1 level, station numbers 1-6). Lymphadenectomy is classified as D2 according to the guidelines of the Japanese Gastric Cancer Association [24]. D2 lymphadenectomy involves the en-bloc removal of lymph node stations 7, 8a and 8p, 9, and 11p and 11d. The left gastric artery was suture ligated at its origin. Lymphadenectomy of the splenic hilum (station 10) was always performed. Hepatoduodenal ligament nodes (station numbers 12a, 12b, 12p) were also dissected. Cholecystectomy was performed in all patients. The resection was extended to the distal esophagus when required by tumor spread and location, which was the case in nearly all of the tumors located at the GEJ. Each lymph node station was removed and classified either during the operation or from the surgical specimen; single lymph nodes were retrieved in the fresh specimen and then submitted to histopathological examination.

For reconstruction, the Roux-en-Y technique was performed in all cases. After total gastrectomy, esophagojejunostomy, using an EEA stapler (diameter 25 mm) was used routinely. In case of a subtotal gastrectomy, Roux-en-Y gastrojejunostomy was performed using an EEA stapler (diameter 25 mm) or a linear stapler (60 mm), at surgeon's discretion. A trans-anastomotic tube was placed in all patients.

Pathological data

The surgical specimens and lymph nodes were assessed by pathologists and were classified according to the 8th Edition of the UICC/AJCC TNM staging system[8]. The T category was used to assess the depth of invasion. For nodal staging, involvement of lymph nodes was defined as follows: N0, no regional lymph nodes metastasis; N1, metastasis in 1 to 2 regional nodes; N2, metastasis in 3 to 6 regional lymph nodes; N3a, metastasis in 7 to 15 regional lymph nodes; N3b, metastasis in > 16 regional lymph nodes. Based on definitive pathological findings, the potentially curative procedures were classified as radical (R0 - microscopic tumor free) or as R1 (microscopic residual disease) according to the absence or presence of residual tumor. Palliative resection was classified based on R2 (macroscopic disease left behind)[24]. Frozen sections were not routinely used in the evaluation of margins, but only in the suspicion of a possible tumor infiltration.

Postoperative course

Antibiotic prophylaxis was used in all patients. Low molecular weight heparin treatment was used in all patients for 30 d. All patients were mobilized on the first postoperative day. The bladder catheter was removed on the first postoperative day except in clinical emergencies. The ERAS protocol was not used in any patient. The anastomosis was routinely checked prior to the patient resuming oral intake with a radiological examination using water-soluble contrast on postoperative day 4-7. The trans-anastomotic tube was removed after performing the radiological control if no sign of anastomotic leak was observed. The patients were monitored for 30 d postoperatively for complications and mortality. Complications were considered when occurring within 30 d from surgery, and with a Clavien-Dindo severity grade 2 or more[25]. Anastomotic leakage was defined as a full thickness gastrointestinal defect involving esophagus, anastomosis, staple line, gastric or jejunal stump irrespective of presentation or method of identification; an abscess close to the anastomoses is also considered as anastomotic leakage.

The patients follow up was standardized as follows: clinical examination, full blood tests and dosage of tumor markers, chest X-ray and abdominal ultrasound every 3 mo for the first 2 years and every 6 mo for the following 3 years. Digestive endoscopy and total-body CT scan were performed annually, unless otherwise required. The evaluation of the nutritional status was managed by specialized nutritionists. No patients were lost to follow-up procedure. All patients with positive lymph nodes were treated with systemic adjuvant chemotherapy.

Statistical analysis

The clinicopathological characteristics included the patient age, sex, resection type, associated splenectomy, tumor site, histological type, T category, N stage, number of lymph nodes examined, number of metastatic lymph nodes, stage of disease, depth of the primitive tumor and Kattan score. Data are expressed as a mean ± SD. Data were analyzed with standard statistical methods using GraphPad Prism Software (GraphPad, CA, United States). Comparison of means ± SD was performed with the two tailed *t*-test. A univariate analysis with all the demographic data and pathologic factors using the Fisher's exact test for categorical data and the ANOVA test for continuous data was performed. Subsequently, a multivariate logistic regression was

performed. Regardless of the used test, a P value < 0.05 was considered statistically significant.

RESULTS

During the study period, a total of 304 patients with gastric cancer were treated at the General Surgery Operative Unit, Fondazione Policlinico Universitario "A Gemelli" IRCCS of Rome, and at the General Surgery Operative Unit, San Giovanni di Dio Hospital, Azienda Sanitaria Provinciale of Crotone. Among them, 186 patients (61.2%) underwent a macroscopic potentially curative D2 lymphadenectomy (R0 resection) and were retrospectively analyzed for this observational study. The other 118 patients were excluded from the evaluation for the presence of distant metastases (50 cases, 16.4%), peritoneal carcinosis (44 cases, 14.4%) diagnosed preoperatively either by laparoscopy (31 cases) or by exploratory laparotomy, or due to R2 surgery (24 cases, 7.9%).

Demographics and intraoperative data

The main demographic data and clinical characteristics of all patients are reported in Table 1. One hundred and eight patients were male (58.1%) and 78 females (41.9%). The mean age was 64.9 ± 12.4 years (range: 24-90 years). One hundred and six patients were older than 65 years (57.0%) and 80 less than or equal to 65 years (43.0%). The mean tumor size was 4.4 ± 2.3 cm (range 0.5-14 cm). With regards to tumor localization a higher percentage of tumors were in the middle or lower third (31.2% and 43.5%, respectively) of the stomach. As far as UICC/AJCC stage groupings, 95 patients (51.0%) were in early stage of the disease (stage IA, IB, IIA) and 91 patients (49.0%) had advanced disease (stage IIB, IIIA, IIIB, IIIC). Only 40 patients (T1a 36 cases - 19.3%, T1b 4 cases - 2.1%) had early gastric cancer (Table 1). Kattan score was 117.8 ± 45.7 points (range 11-215).

Total gastrectomy was performed in 88 patients (47.3%) and subtotal gastrectomy in 98 (52.7%). Mean age of patients undergoing total gastrectomy was 63 ± 12.1 years and 66.6 ± 12.5 years in those undergoing subtotal gastrectomy (P = 0.04). In the total gastrectomy patient's subgroup, the mean Kattan score was 111.3 ± 44.1 points, statistically lower (P = 0.03) than that observed after subtotal gastrectomy (125.1 ± 46.7 points). The mean tumor size was 4.6 ± 2.6 cm (range 1-14) and 4.1 ± 2.0 cm (range 0.5-11) in patients undergoing total gastrectomy and subtotal gastrectomy, respectively (P = 0.1).

To obtain an R0 resection, adjacent organs were removed in 5 patients (2.7%): in two cases an atypical liver resection was performed, and in 3 a transverse colon resection was performed. A mean number of 38.3 ± 10.9 lymph nodes (range 17-98) were dissected. The average number of positive lymph nodes was 4.2 ± 6.3 (range 0-39). 74 patients were N0. The mean number of lymph nodes removed was 40 ± 10.4 (range 25-93) and 36.7 ± 11.1 (range 17-98) in total gastrectomy and subtotal gastrectomy, respectively (P = 0.03). The number of positive lymph nodes was 4.9 ± 6.9 (range 0-39) in patients undergoing total gastrectomy and 3.5 ± 5.7 (range 0-31) in patients undergoing subtotal gastrectomy (P = 0.1). Lymphadenectomy of the splenic hilum involved splenectomy in 105 cases (56.4%) and was performed with the spleen-preserving technique in the remaining 81 cases (43.6%). 103 patients (55.3%) had > 35 lymph nodes retrieved. Mean duration of surgical procedures was 260 ± 76.1 minutes. Mean length of postoperative hospital stay was 12.7 ± 8.2 d.

Mortality

Perioperative mortality rate was 3.8% (7 patients). Causes of death were pancreatic fistula (2 cases), hemoperitoneum (2 cases, one of which was associated with a pancreatic fistula), dehiscence of the esophago-jejunal anastomosis (1 case), dehiscence of the duodenal stump (2 cases) and aspiration pneumonia resulting in ARDS (1 case). A higher mortality was observed in the group of patients aged > 65 years (7 cases out of 80, 8.7%) compared to those aged < 65 years (no cases in 106 patients, P = 0.002) and in N + patients (7 cases out of 112, 6.2%) compared to N- patients (no cases out of 74 patients, P = 0.04, Table 2).

In the univariate analysis a significant mortality rate was observed in the group of patients aged > 65 years (P = 0.008), in patients with Kattan score ≥ 100 points (P = 0.04), and in patients with advanced gastric cancer (P = 0.03). Sex (P = 0.4), type of surgery performed (P = 0.8), primary tumor location (P = 0.8), tumor depth (P = 0.1), and Lauren histological type (P = 0.4) had no statistically significant influence on

Table 1 Clinico-pathologic patient characteristics

Characteristics		
Sex		
Male	108	58.1%
Female	78	41.9%
Age, yr	64.9 ± 12.4	Range 24-90
Primary tumor location		
Gastroesophageal junction	22	11.8%
Upper third	25	13.4%
Middle third	58	31.2%
Lower third	81	43.5%
Histological type (Lauren classification)		
Enteric type	96	51.6%
Diffuse type	64	34.4%
Mixed type	26	14.0%
Type of resection		
Total gastrectomy	88	
Subtotal gastrectomy	98	
Size, cm	4.4 ± 2.3	Range 0.5-14
Number of lymph nodes retrieved	38.3 ± 10.9	Range 17-98
Number of positive lymph nodes	4.1 ± 3.6	Range 0-39
Operation time, minutes	260 ± 76.1	
Length of stay, d	12.7 ± 8.2	
T status		
T1a	36	19.3%
T1b	4	2.1%
T2	79	42.5%
T3	56	30.1%
T4a	2	1.1%
T4b	9	4.8%
Depth		
Mucosa	14	7.5%
Submucosa	31	16.7%
Muscularis propria	37	19.9%
Subserosa (suspected invasion)	46	24.7%
Subserosa (certain invasion)	49	26.3%
Serosa	4	2.1%
Adjacent structures	5	2.7%
N status		
N0	74	39.8%
N1	37	19.9%
N2	31	16.7%
N3a	33	17.7%

N3b	11	5.9%
Stage AJCC/TNM		
IA	29	15.6%
IB	32	17.2%
IIA	34	18.3%
IIB	35	18.8%
IIIA	23	12.4%
IIIB	26	14.0%
IIIC	7	3.8%

Values are mean ± SD. All the patients were included in all evaluations.

perioperative mortality (Table 3). In the multivariate analysis (Table 3) only age > 65 years had a statistically significant influence (T ratio 2.960, $P = 0.004$) on perioperative mortality.

Postoperative overall complications

Postoperative complications were documented in 40 patients (21.5%). Table 4 Lists the type of complications and their frequency. As shown, pulmonary complications, urinary tract infections, pancreatic fistulas, anastomotic leaks and duodenal fistula were the most frequently observed complications.

A higher incidence of complications was observed in patients undergoing subtotal gastrectomy (29 cases out of 98 patients, 29.5%) compared to those undergoing total gastrectomy (11 cases out of 88 patients, 12.5% - $P = 0.006$), in patients with Kattan score ≥ 100 points (32 cases out of 121 patients, 26.4%) compared to those with Kattan score < 100 points (8 out of 65 patients, 12.3% - $P = 0.02$) and in those N + (30 out of 112 patients, 26.7%) compared to those N- (10 of 74 patients, 13.5% - $P = 0.04$, Table 2).

Univariate analysis (Table 5) confirmed that sex, age, number of lymph nodes harvested, primary tumor site and histological type are not related to morbidity. This is related to the type of surgery ($P = 0.005$), the Kattan score ($P = 0.02$), the tumor depth ($P = 0.01$), T stage ($P = 0.006$) and the stage of the disease ($P = 0.01$). In the multivariate analysis (Table 5) only the extent of surgery showed a statistically significant correlation (T ratio 2.526, $P = 0.01$).

Postoperative surgical complications

Surgical complications were observed in 17 patients (9.1%). Among these, the most frequent were duodenal fistula (5 cases), pancreatic fistula (4 cases, one of which associated with hemoperitoneum) and dehiscence of the esophago-jejunal anastomosis. Four patients (2 cases of hemoperitoneum, 2 cases of duodenal fistula) underwent further surgical treatment. The two patients with bowel obstruction underwent adhesion lysis surgery 2 mo and 6 mo after gastric surgery, respectively. All other patients with surgical complications were treated conservatively. A higher incidence of surgical complications was observed in the patient group with more than 35 lymph nodes harvested (16 cases out of 103 patients, 15.5%) compared to patients in which fewer lymph nodes were removed (1 case in 83 patients, 1.2% - $P = 0.0005$). Sex ($P = 0.7$), age > 65 years ($P = 0.2$), type of surgery performed ($P = 0.6$), Kattan score ($P = 0.1$), lymph node positivity ($P = 0.1$) and early stage of disease ($P = 0.5$) did not affect the rate of perioperative surgical complications (Table 2).

This was confirmed by the univariate analysis, which documented that the removal of more than 35 lymph nodes ($P = 0.002$), the depth of the tumor ($P = 0.04$) and the stage of disease ($P = 0.01$) are statistically correlated with the development of surgical complications in the postoperative period (Table 6).

On multivariate analysis (Table 6) only one lymphadenectomy with removal of more than 35 lymph nodes correlates significantly with the rate of surgical complications (T ratio 3.222, $P = 0.001$).

Table 2 Mortality, overall morbidity and surgical morbidity in all patients

Characteristics	Number of cases	Mortality	Overall morbidity	Surgical morbidity
	186	7	40	17
Sex				
Male	108	3	24	9
Female	78	4	16	8
		$P = 0.4$	$P = 0.8$	$P = 0.7$
Age				
> 65 yr	80	7	22	10
≤ 65 yr	106	0	18	7
		$P = 0.002$	$P = 0.1$	$P = 0.2$
Type of surgery				
TG	88	3	11	7
STG	98	4	29	10
		$P = 1.0$	$P = 0.006$	$P = 0.6$
Kattan score				
≥ 100 points	121	7	32	14
< 100 points	65	0	8	3
		$P = 0.09$	$P = 0.02$	$P = 0.1$
Lymphadenectomy				
> 35 lymph nodes	103	3	24	16
≤ 35 lymph nodes	83	4	16	1
		$P = 0.7$	$P = 0.5$	$P = 0.0005$
Lymph nodes				
Negative	74	0	10	4
Positive	112	7	30	13
		$P = 0.04$	$P = 0.04$	$P = 0.1$
T				
Early cancer	40	0	5	2
Advanced cancer	146	7	35	15
		$P = 0.3$	$P = 0.1$	$P = 0.5$
Splenectomy	105	4	24	9
Spleen-preserving	81	3	16	8
		$P = 1.0$	$P = 0.7$	$P = 0.8$

TG: Total gastrectomy; STG: Subtotal gastrectomy. All the patients were included in all evaluations. Fisher exact test two-tailed.

DISCUSSION

Surgical treatment is still the mainstay of curative gastric cancer treatment[4,26-29]. For patients who undergo surgery, prognosis is determined by a series of factors, among which depth of invasion, nodal status, and metastasis are the most important. These factors are part of the UICC/AJCC stage formula, which is the most reliable prognostic system. In addition, certain multivariate analyses could identify extent of lymphadenectomy, lymph node ratio (ratio between positive and removed nodes), residual tumors, and grading, as independent prognostic factors. The expected prognosis has great impact on the kind of treatment a patient will receive. The

Table 3 Univariate and multivariate analysis of variables associated with postoperative mortality

	Univariate analysis			Multivariate analysis	
	Number of cases	%	*P* value	T ratio	*P* value
Sex (Male)	3	42.9	0.406	0.5888	0.557
Age > 65 yr	7	100	0.008	2.960	0.004
Type of surgery (subtotal gastrectomy)	4	57.1	0.810		
Kattan score ≥ 100	7	100	0.048	0.9504	0.343
Lymph nodes > 35	6	85.7	0.152	1.745	0.114
Primary site			0.821		
Gastroesophageal junction	1	14.3			
Upper third	1	14.3			
Middle third	1	14.3			
Lower third	4	57.1			
Depth			0.137	1.231	0.220
Mucosa	0				
Submucosa	1	14.3			
Muscularis Propria	0				
Subserosa (suspected invasion)	2	28.6			
Subserosa (certain invasion)	3	42.9			
Serosa	0				
Adjacent structures	1	14.3			
Histological type (Lauren classification)			0.436		
Enteric type	3	42.9			
Diffuse type	2	28.6			
Mixed type	2	28.6			
T status			0.031	1.342	0.181
T1a	0				
T1b	0				
T2	3	42.9			
T3	2	28.6			
T4a	0				
T4b	2	28.6			
Stage AJCC/TNM			0.039	0.6371	0.525
IA	0				
IB	0				
IIA	1	14.3			
IIB	2	28.6			
IIIA	1	14.3			
IIIB	3	42.9			
IIIC	0				

standard for nodal staging of gastric cancer has international variation, and recently significant changes have been made to the AJCC/UICC staging system to simplify lymph node staging in the countries using TNM staging. In the most recent AJCC edition N1 represents 1-6 positive lymph nodes; N2 represents 7-15 positive lymph

Table 4 Major postoperative complications with a severity grade 2 or more according Clavien-Dindo classification

Type of complication	Number of cases	%
Pulmonary	12	6.4
Urinary tract infection	10	5.4
Leak of esophago-jejunal anastomosis	4	2.1
Intra-abdominal abscess	1	0.5
Abdominal bleeding	2	1.0
Duodenal fistula	5	2.7
Intestinal occlusion	2	1.0
Pancreatic fistula	4	2.1

nodes; and N3 represents > 15 positive lymph nodes. The cut-off points were determined from retrospective databases[30] and in subsequent evaluations showed a superior predictive ability compared to other staging systems[31,32].

The extent of lymphadenectomy is the only factor that can be influenced by the surgeon[33-38]. The total number of lymph nodes resected, or the total number of positive to negative ratio of lymph nodes have all been found to be predictors of survival in gastric cancer patients[37]. For potentially resectable gastric cancer, a linear trend toward superior survival was found for higher lymph node removal up to 35-40 lymph nodes, based on the analysis of the SEER database from 1973 to 1999[38]. Adjuvant therapy is used in advanced gastric cancer to improve the survival and may be useful in high-risk patients treated with limited lymph node dissection. Moreover, lymph node dissection remains crucial to make every effort to improve the prognosis in those patients unsuitable for any adjuvant treatment[39,40]. In a study Biffi *et al*[13] showed that extended lymph node resection offers survival benefit even in the subgroup of patients with early-stage disease. Evaluation of distant disease-free survival risk by number of harvested lymph nodes showed that the risk of recurrence is inversely proportional to the number of dissected lymph nodes. The results did not change when pT1 and pT2-3 cancers were analyzed separately, suggesting the need to remove at least 15 nodes even in patients with early-stage disease[13].

The idea of an extended lymphadenectomy for gastric cancer was first advanced by Mikulicz in 1889, who stated that the distal pancreas should be removed if necessary [40-42]. Recent studies show that D2 lymphadenectomy improves the accuracy of locoregional staging and might reduce disease recurrence in patients with gastric adenocarcinoma[27]. Furthermore, when expert surgeons perform D2 lymphadenectomy and avoid routine distal pancreatectomy and splenectomy, perioperative morbidity and mortality can be kept to a minimum[43,44].

Although neither the 5-year[28] nor 11-year results[40] of the Dutch trial showed a significant improvement in overall survival for patients randomized to D2 lymphadenectomy compared with D1, we believe that surgery remains the only non-standardized therapy in the context of clinical trials and that D2 resection has clinical relevance in most treatment algorithms. Several surgeons agree that standardized D2 lymphadenectomy is an appropriate and potentially beneficial treatment approach[45,46]; like any therapy, surgery must be done safely and correctly by skilled clinicians and should be tailored to the patient and biology of the disease[4,47,48].

Marubini *et al*[10] examined 615 resections, and found no difference in mortality (1.8%) or complication rates (12.8%) with respect to the number of harvested nodes, but better overall survival when more lymph nodes were assessed. With more than 11 years of median follow-up, there was a trend for improved survival for patients with N2 disease who had received a D2 dissection[40]. Another analysis excluding patients with distal pancreatectomy and splenectomy found a survival benefit for the D2 resection patients[49]. Clinical series from Asia have found a low rate of nodal recurrences following aggressive lymph nodes dissection. Furthermore, Japanese investigators have recently completed trials of D2 *vs* D2 plus para-aortic nodal dissection, showing better results in small cancer with negative nodes who underwent aggressive D2 dissection[4]. Moreover, if D2 lymphadenectomy was performed, it was likely to have a marked benefit compared to D1 dissection[14,50].

Despite the therapeutic value of lymphadenectomy, mortality and complications are still high in gastric cancer surgery[16,51]. Several studies point out that stomach cancer

Table 5 Clinicopathological factors associated with overall morbidity by univariate and multivariate analysis

	Univariate analysis			Multivariate analysis	
	Number of cases	%	*P* value	T ratio	*P* value
Sex (Male)	24	60	0.779	0.8443	0.4
Age > 65 yr	22	55	0.575	0.4271	0.670
Type of surgery (subtotal gastrectomy)	29	72.5	0.005	2.526	0.012
Kattan score ≥ 100	32	80	0.026	0.5097	0.611
Lymph nodes > 35	24	60	0.962		
Primary site			0.180	0.3756	0.708
Gastroesophageal junction	5	12.5			
Upper third	1	2.5			
Middle third	7	17.5			
Lower third	27	67.5			
Depth			0.017	0.2270	0.821
Mucosa	1	2.5			
Submucosa	5	12.5			
Muscularis propria	5	12.5			
Subserosa (suspected invasion)	11	27.5			
Serosa	3	7.5			
Adjacent structures	4	10.0			
Histological type (Lauren classification)			0.265	0.4180	0.677
Enteric	17	42.5			
Diffuse	13	32.5			
Mixed	10	25.0			
T status			0.006	0.6177	0.538
1a	0				
1b	5	12.5			
2	15	37.5			
3	12	30.0			
4a	4	10.0			
4b	4	10.0			
Stage AJCC/TNM			0.018	0.8390	0.403
IA	5	12.5			
IB	2	5.0			
IIA	6	15.0			
IIB	10	25.0			
IIIA	7	17.5			
IIIB	8	20.0			
IIIC	2	5.0			

AJCC: American Joint Committee on Cancer.

surgery is a complex procedure that leads to a high risk of morbidity and mortality [15]. Li *et al*[52] observed 30 d and 90 d mortality of 2.0% and 3.4%, respectively, in patients undergoing total gastrectomy for cancer. These data are consistent with what

Table 6 Factors associated with surgical complications in univariate and multivariate analysis

	Univariate analysis			Multivariate analysis	
	Number of cases	%	*P* value	T ratio	*P* value
Male sex	9	52.9	0.653	0.4193	0.675
Age > 65 yr	10	58.8	0.502	1.192	0.235
Type of surgery (subtotal gastrectomy)	10	58.8	0.595		
Kattan score ≥ 100	14	82.3	0.116	0.08543	0.932
Lymph nodes > 35	16	94.1	0.002	3.222	0.001
Primary site			0.609		
Gastroesophageal junction	4	23.5			
Upper third	1	5.9			
Middle third	2	11.8			
Lower third	10	58.8			
Depth			0.045	0.8208	0.413
Mucosa	0				
Submucosa	2	11.8			
Muscularis Propria	2	11.8			
Subserosa (suspected invasion)	5	29.4			
Subserosa (certain invasion)	3	17.6			
Serosa	2	11.8			
Adjacent structures	3	17.6			
Histological type (Lauren classification)			0.817		
Enteric type	8	47.1			
Diffuse type	4	23.5			
Mixed type	5	29.4			
T status			0.054	1.102	0.272
T1a	0				
T1b	2	11.8			
T2	7	41.2			
T3	4	23.5			
T4a	1	5.9			
T4b	3	17.6			
Stage AJCC/TNM			0.019	0.8237	0.411
IA	1	5.9			
IB	0				
IIA	3	17.6			
IIB	5	29.4			
IIIA	3	17.6			
IIIB	4	23.5			
IIIC	1	5.9			

AJCC: American Joint Committee on Cancer.

is reported by other authors. Selby *et al*[53] reported data of 2.5% and 2.9% at 30 d and 90 d, respectively, while Pacelli *et al*[54] reported a mortality of 3.5% in 312 patients undergoing potentially curative gastrectomy for cancer. We observed a perioperative mortality rate of 3.8%. A higher mortality was observed in the group of patients aged > 65 years (8.7%) and in N + patients (6.2%).

The risk of postoperative complications is also high. Li *et al*[52] reports a complication rate of 43.9%, with a 14% incidence of severe (class III and class IV according to the Clavien-Dindo classification) complications. A severe complication after total gastrectomy is the anastomotic leak of the esophagojejunal anastomosis. In our experience, dehiscence occurred in 4 patients (2.1%), and was fatal in one case. Selby *et al*[53] and Pacelli *et al*[54] report an incidence of anastomotic dehiscence of 14.7% and 8.6% respectively. In our experience, all anastomotic leakages were identified in the early postoperative period, from day 4 to day 7, by performing routine upper GI contrast studies. The anastomotic leak leads to an increase in the duration of hospitalization, with increases ranging from 13 to 48 d of hospitalization [55]. Another severe complication is duodenal stump dehiscence. This complication occurred in 5 of our patients (2.7%), representing the cause of death in two of them. This complication also increased mortality in the literature[56]. We observed 2 cases of hemoperitoneum (1.0%) and 4 cases of pancreatic fistula (2.1%). These complications were fatal in the two cases of hemoperitoneum and in two of the 4 cases of pancreatic fistula. They were only observed in the patient group where more than 35 lymph nodes had been removed. In our series, mortality occurred only in the group of patients with a higher Kattan score. It seems likely that advanced stage tumors may alter the responsiveness of the patient, increasing the incidence of complications and mortality.

In our study, the overall incidence of surgery-related complications was 9.1%. As easy to predict, morbidity rate is higher in advanced tumors than in the earlier stage. The overall morbidity rate is higher in patients with Kattan score ≥ 100 (P = 0.02) and in N + patients (P = 0.04). Contrary to what has been observed in the literature, we documented a higher morbidity rate in patients undergoing subtotal gastrectomy (29 cases *vs* 11 cases after total gastrectomy - P = 0.006). We believe that this is related to a higher mean age in patients who underwent subtotal gastrectomy (66.6 ± 12.5 years, range: 24-90) than in those who underwent total gastrectomy (63 ± 12.1 years, range: 30-84, P = 0.04), and a higher mean Kattan score (125.1 ± 46.7 points, range 11-206) than in patients who underwent total gastrectomy (111.3 ± 44.1, range 24-215, P = 0.03). We observed a higher prevalence, without statistical significance (P = 0.2), of patients with Kattan ≥ 100 points in the group undergoing subtotal gastrectomy (64 patients, 65.3%) compared to those undergoing total gastrectomy (49 cases, 55.6%). Regarding other parameters considered, such as the size of the tumor (4.1 ± 2.0 cm in subtotal gastrectomy *vs* 4.6 ± 2.6 cm in total gastrectomy, P = 0.1), the average number of positive lymph nodes (3.5 ± 5.7 in subtotal gastrectomy *vs* 4.6 ± 2.6 in total gastrectomy, P = 0.1) we did not find statistically significant differences. The number of lymph nodes removed was higher in patients undergoing total gastrectomy (40 ± 10.4) than in those undergoing subtotal (36.7 ± 11.1, P = 0.03).

A higher incidence of surgical complications was observed in patients in whom more than 35 lymph nodes were removed. This data was confirmed in the univariate and multivariate analyses, where lymphadenectomy with the removal of more than 35 lymph nodes is the only factor that shows correlation with surgical complications. We have documented two cases of hemoperitoneum and 4 pancreatic fistulas, all in patients with spleen-preserving lymphadenectomy. Performing splenectomy for station 10 lymphadenectomy did not in our experience induce an increase in mortality and morbidity. These complications were found to be severe, as reported in the literature[26,57,58]. Many studies show that risk factors for the development of pancreatic fistula are the weight of the patient, the anatomy and texture of the pancreas, intraoperative trauma of the pancreas and the use of high-energy devices when performing lymphadenectomy[26,57].

Although we are aware that the Kattan nomogram was created to evaluate the long-term prognosis and survival of patients with gastric cancer undergoing R0 resection, we observed that the Kattan score, at the cut-off value used, is useful as a prognostic index even in the early postoperative phase. In our experience only patients with Kattan score ≥ 100 points died; a good correlation was also documented as far as the complication rate. Since Kattan takes into account, in addition to age, many characteristics of the tumor and the lymph node status, we have documented, as reported in the literature, that the incidence of mortality and major complications are observed with greater frequency in elderly patients, with more advanced and N + stage cancers. An intrinsic difficulty in using the Kattan score is the fact that the score itself is based on a

lot of histopathological information which are not always readily available.

All our patients underwent cholecystectomy. The procedure did not cause biliary complications. This aspect is controversial in the literature. In patients with a radical resection, when a D2 lymphadenectomy is performed and the duodenum is excluded in the intestinal reconstruction, cholecystectomy, considered by some to be a non-essential measure, is necessary to avoid gallstone formation and its complications. In this setting, we believe that prophylactic cholecystectomy is necessary for patients with a good cancer prognosis, as suggested by Pitt and Nakeeb[59]. Studies on the subject conclude that prophylactic cholecystectomy does not have a significant impact on the natural course of the disease[60]. However, it leads to a reduction in the number of biliary complications (which may affect up to 15% of the operated patients) and does not induce an increase in mortality and morbidity rates. In one study, a mortality rate of 1.8% was reported in the case of cholecystectomy performed during an intervention after a gastrectomy. Prophylactic cholecystectomy seems to be unnecessary only in cases where the continuity of the digestive tract involves the use of the duodenum[61]. It was found that the method used to restore intestinal continuity, with preservation of the duodenal transit or excluding the duodenum, is an independent risk factor for both the development of cholelithiasis (P = 0.018) and cholecystitis and cholangitis (P = 0.006). It has also been confirmed that in patients who develop cholelithiasis, the incidence of cholecystitis and cholangitis is particularly high when the duodenal transit is excluded (31.3%) compared to those with maintained duodenal transit (7.4%).

CONCLUSION

It is fair to reiterate that gastric cancer surgery is a complex surgical procedure. Mortality and postoperative complications are linked both to the extent of gastric demolition and to lymphadenectomy. In our experience, the removal of more than 35 lymph nodes conditioned an increase in surgical complications, although it did not lead to an increase in mortality. Mortality was higher in elderly patients, N + patients and patients with advanced gastric cancer. These parameters (age, T status and N status) are included in the Kattan score, which can be useful, if the histopathological parameters can be obtained quickly, as a prognostic tool even in the early phase.

ARTICLE HIGHLIGHTS

Research background

Gastric cancer surgery is a complex procedure. Lymphadenectomy is essential for the surgical treatment of gastric cancer. Mortality and postoperative morbidity after gastric cancer surgery are not negligible.

Research motivation

We investigated in a population of 186 patients with stomach cancer undergoing surgery with D2 lymphadenectomy which factors were related to postoperative mortality and morbidity.

Research objectives

To evaluate the factors determining mortality and morbidity in a population of patients undergoing R0 resection and D2 lymphadenectomy for gastric cancer.

Research methods

For each patient we calculated the Kattan's score. The following prognostic variables were assembled for use in validating the nomogram: age, sex, primary site (distal one-third, middle one-third, proximal one-third, and gastroesophageal junction), Lauren histotype (diffuse, intestinal, mixed), number of positive lymph nodes resected, number of negative lymph nodes resected, and depth of invasion as defined by the standard nomenclature.

Research results

Perioperative mortality rate was 3.8% (7 patients); a higher mortality rate was observed in patients aged > 65 years (P = 0.002) and in N+ patients (P = 0.04).

Following univariate analysis, mortality was related to a Kattan's score ≥ 100 points (P = 0.04) and the presence of advanced gastric cancer (P = 0.03). Morbidity rate was 21.0% (40 patients). Surgical complications were observed in 17 patients (9.1%). A higher incidence of morbidity was observed in patients where more than 35 lymph nodes were harvested (P = 0.0005).

Research conclusions

Mortality and morbidity rate are higher in N+ and advanced gastric cancer patients. The removal of more than 35 lymph nodes does not lead to an increase in mortality.

Research perspectives

An extended lymph nodes dissection in patients undergoing surgical treatment for gastric cancer is a safe procedure.

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World Journal of Gastroenterology

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World J Gastroenterol 2022 January 21; 28(3): 399-401

DOI: 10.3748/wjg.v28.i3.399

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

LETTER TO THE EDITOR

Prophylactic drains in totally laparoscopic distal gastrectomy: are they always necessary?

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Author contributions: Manzia TM and Angelico R designed the research; Parente A wrote the letter; Manzia TM revised the letter; all authors approved the final version of the manuscript.

Conflict-of-interest statement: The authors declare that they have no conflicts of interest to disclose.

Country/Territory of origin: Italy

Specialty type: Gastroenterology and hepatology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

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Abstract

Prophylactic drains have always been a useful tool to detect early complications and prevent postoperative fluid collections, particularly in gastrointestinal surgery. Recently, the utilization of such drains has been debated, due to mounting evidence that they could be harmful rather than beneficial. Based on recent published articles, Liu *et al* reported that the routine use of prophylactic drains in total laparoscopic distal gastrectomy might not be necessary for all patients. Herein, we express our opinion regarding this interesting publication.

Key Words: Gastric cancer; Prophylactic drainage; Totally laparoscopic gastrectomy; Enhanced recovery after surgery; Minimally invasive surgery; Early gastric cancer

Core Tip: Historically, prophylactic drains have been used to prevent postoperative collections and detect complications. In recent decades, there have been increasing reports that debate their routine usage in gastrointestinal surgery. Liu *et al* have shown that prophylactic drains can be safely omitted in selected patients undergoing totally laparoscopic distal gastrectomy. In this letter to the editor, we express our opinion regarding these interesting findings.

Citation: Manzia TM, Parente A, Angelico R. Prophylactic drains in totally laparoscopic distal gastrectomy: are they always necessary? *World J Gastroenterol* 2022; 28(3): 399-401

URL: https://www.wjgnet.com/1007-9327/full/v28/i3/399.htm

Received: July 18, 2021
Peer-review started: July 18, 2021
First decision: October 3, 2021
Revised: October 5, 2021
Accepted: January 11, 2022
Article in press: January 11, 2022
Published online: January 21, 2022

P-Reviewer: Kawabata H
S-Editor: Wang LL
L-Editor: Webster JR
P-Editor: Wang LL

DOI: https://dx.doi.org/10.3748/wjg.v28.i3.399

TO THE EDITOR

We read with great interest the study by Liu *et al*[1]. These authors analyzed the outcome of 125 patients undergoing totally laparoscopic gastrectomy for distal gastric cancer with or without prophylactic drain (PD) insertion. In this retrospective study, Liu *et al*[1] demonstrated that in patients without placement of PDs there was no increased risk of postoperative complications. Furthermore, omitting a PD was associated with greater patient comfort. Of particular note, these interesting findings were confirmed by a propensity score matched analysis of 42 patients with and without PDs.

PDs facilitate the removal of postoperative fluid, which can potentially collect and become infected. In addition, PDs can help identify early postoperative complications such as anastomotic leakage and bleeding. In recent decades, the advances in surgical care have led to an overall decrease in postoperative complications. Therefore, the need for PDs has been debated and there is mounting evidence that they may even increase the risk of complications without preventing the need for reoperation. As an example, in major procedures such as liver resection, it has been shown that PDs increased the rate of biliary leak, length of hospital stay and total complications[2]. The same outcomes were demonstrated in gastric surgery in a recent meta-analysis[3].

This is one of the few studies which highlight the issues of PDs in total laparoscopic distal gastrectomy and, interestingly, the authors identified body mass index (BMI) ≥ 29 kg/m^2 to be associated with a higher risk of postoperative complications. The outcomes illustrated by the authors are in line with similar previously published articles[4,5]. Although the results by Liu *et al* are compelling, they need to be interpreted with caution. The data presented are prospectively maintained and retrospectively reviewed, but it is difficult to estimate the overall burden of postoperative morbidity as minor complications (Grade I), have not been included. Such examples are acute kidney injury treated with intravenous fluids, nausea treated with antiemetics, or electrolyte imbalances that responded to replacement therapy. This would add a more precise evaluation to the role of PDs in the postoperative setting, as minor complications could play an important role especially in the length of hospital stay. Secondly, the decision to insert a PD was made by the operating surgeon and the decision-making process which led to drain placement is unclear. This could bias the data, as it might be related to longer operative times and difficult surgery in high BMI patients. Thirdly, the cohort for this study was from a single-center, hence the generalizability to broader populations cannot be confirmed.

In summary, the authors should be commended for their work. They have demonstrated with a well-conducted analysis that PDs are not an independent risk factor for postoperative complications with the caveat that there appears to be a higher risk in patients with BMI ≥ 29 kg/m^2; therefore, in this group PDs are recommended. Identifying pre-operative and intraoperative factors that can guide the decision-making in order to select low-risk patients with regard to the omission of PDs would be of great interest. Furthermore, randomized controlled trials on PDs *vs* non-PDs insertion, focusing on laparoscopic approaches for gastric surgery, would be useful to guide clinical decisions.

ACKNOWLEDGEMENTS

The authors express their gratitude to Dr. Hann A from University Hospitals Birmingham, United Kingdom, for his invaluable contribute to the English editing of this manuscript.

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